METHODS FOR MODULATING AN IMMUNE RESPONSE BY MODULATING KRC ACTIVITY

Related Applications

This application is a continuation-in-part of U.S. application No. 10/701,401, filed November 3, 2003, which claims the benefit of priority to PCT application PCT/US02/14166, filed May 3, 2002, and U.S. Provisional Application Serial No. 60/288,369, filed May 3, 2001. The entire contents of each of these applications are incorporated herein by this reference.

Background of the Invention

Transcription factors are a group of molecules within the cell that function to connect the pathways from extracellular signals to intracellular responses. Immediately after an environmental stimulus, these proteins which reside predominantly in the cytosol are translocated to the nucleus where they bind to specific DNA sequences in the promoter elements of target genes and activate the transcription of these target genes. One family of transcription factors, the ZAS (zinc finger-acidic domain structures) DNA binding protein family is involved in the regulation of gene transcription, DNA recombination, and signal transduction (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39).

Zinc finger proteins are identified by the presence of highly conserved Cys2His2 zinc fingers (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39). The zinc fingers are an integral part of the DNA binding structure called the ZAS domain. The ZAS domain is comprised of a pair of zinc fingers, a glutamic acid/aspartic acid-rich acidic sequence and a serine/threonine rich sequence (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39). The ZAS domains have been shown to interact with the kB like *cis*-acting regulatory elements found in the promoter or enhancer regions of genes. The ZAS proteins recognize nuclear factor kB binding sites which are present in the enhancer sequences of many genes, especially those involved in immune responses (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648). The ZAS DNA binding proteins have been shown to be transcription regulators of these target genes (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001).

The zinc finger transcription factor Kappa Recognition Component ("KRC") is a member of the ZAS DNA binding family of proteins (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001). The KRC gene was identified as a DNA binding protein for the heptameric consensus signal sequences involved in somatic V(D)J recombination of the immune receptor genes (Mak, C. H., *et al.* 1994. *Nuc.Acid Res. 22:* 383-390). KRC is a substrate for epidermal growth factor receptor kinase and p34cdc2 kinase in vitro (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648). However, other functions of KRC and the signal transduction pathways that activate KRC in vivo were not known.

Gene-specific transcription factors provide a promising class of targets for novel therapeutics because they provide substantial specificity and are known to be involved in human disease. A number of extremely effective presently marketed drugs act, at least indirectly, by modulating gene transcription. For instance, in many cases of heart disease, the LDL receptor is pathogenically down-regulated at the level of transcription by intracellular sterol levels. The drug compactin, an inhibitor of HMG CoA reductase, functions by up-regulating transcription of the LDL receptor gene which leads to clearance of cholesterol from the blood stream.

In another example, transcription factors can be modulated to regulate an immune response. In autoimmune diseases, self-tolerance is lost and the immune system attacks "self" tissue as if it were a foreign target. Many autoimmune diseases are presently known, such as multiple sclerosis (MS), rheumatoid arthritis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, Crohn's disease, Guillain-Barre syndrome, psoriasis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, etc. In diseases such as these, inhibiting the immune response is desirable. In addition, inhibiting the body's immune response is beneficial in prevention, for example, of organ transplant rejection. Conversely, enhancing the immune response is beneficial in certain circumstances such as the treatment of AIDS, cancer, atherosclerosis and diabetic complications (Sen, P. *et al.* 1996. *FASEB Journal* 10:709-720, 1996). Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. Specifically, agents for use modulating such cellular processes in T cells are needed to regulate the immune

response. Agents and methods of using such agents in modulation of cell survival, proliferation, differentiation and/or motility would be of great benefit.

Summary of the Invention

The present invention is based, at least in part, on the discovery that KRC molecules have multiple important functions as modulating agents in regulating a wide variety of cellular processes. The invention is based, at least in part, on the discovery that KRC inhibits NFkB transactivation, increases TNF-alpha induced apoptosis, inhibits JNK activation, inhibits endogenous TNF-alpha expression, promotes immune cell proliferation and immune cell activation (*e.g.*, in T cells (such as Th1 and/or Th2 cells), B cells, or macrophages), activates IL-2 expression *e.g.*, by activating the AP-1 transcription factor, and increases actin polymerization. The present invention also demonstrates that KRC interacts with TRAF. Furthermore, the present invention demonstrates that KRC physically interacts with the c-Jun component of AP-1 to control its degradation. The present invention also demonstrates that KRC is downstream of several lymphocyte membrane receptors, including TNFR, TCR and TGFβR. Upon TNFR signaling, KRC associates with the adaptor protein TRAF2 to inhibit NFκB and JNK-dependent gene expression. Upon TCR stimulation, KRC expression is rapidly induced and KRC physically associates with the c-Jun transcription factor to augment AP-1 dependent gene transcription. KRC knock-out (KO) T cells have impaired production of AP-1-dependent genes such as CD69 and IL-2. Upon TCR stimulation KRC also associates with the Th2-specific transcription factor GATA3, and T cells lacking KRC have impaired production of GATA3 dependent Th2 cytokines, IL-4, IL-5 and IL-13. Finally, upon TGFβ receptor signaling, KRC physically associates with the transcription factor SMAD3 to activate IgA germline transcription in B cells, since KRC KO B cells have impaired IgA production and germline Igα (GLα) gene transcription.

In one aspect, the invention pertains to a method for identifying a compound which modulates an interaction between a first and a second polypeptide comprising: (a) contacting a cell having a first polypeptide comprising a binding portion of a KRC polypeptide and a second polypeptide comprising a binding portion of a polypeptide selected from the group consisting of: Jun, GATA3, SMAD, or Runx2 in the presence and the absence of a test compound; and (b) determining the degree of

interaction between the first and the second polypeptide in the presence and the absence of the test compound, to thereby identify a compound which modulates an interaction between a first and a second polypeptide.

In one embodiment, the first polypeptide comprises at least one KRC zinc finger domain. In one embodiment, the second polypeptide is a c-Jun polypeptide. In another embodiment, the second polypeptide is a SMAD2 polypeptide. In another embodiment, the second polypeptide is a SMAD3 polypeptide.

In one embodiment, the first polypeptide is derived from an exogenous source. In another embodiment, the second polypeptide is derived from an exogenous source.

In one embodiment, the cell is a yeast cell.

In one embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the compound to modulate growth of the yeast cell on nutritionally selective media.

In another embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the compound to modulate expression of a reporter gene in the yeast cell.

In one embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the test compound to modulate the coimmunoprecipitation of the first polypeptide and the second polypeptide.

In another embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the test compound to modulate signaling via a signal transduction pathway involving KRC in the cell.

In one embodiment, at least one of TNFα production, IL-2 production, AP-1 activity, Ras and Rac activity, actin polymerization, ubiquitination of AP-1, ubiquitination of TRAF, ubiquitination of Runx2, degradation of c-Jun, degradation of c-Fos degradation of SMAD, degradation of Runx2, degradation of GATA3, GATA3 expression, Th2 cell differentiation, Th2 cytokine production, IgA production, GLα

transcription (Igα chain germline transcription), and/or osteocalcin gene transcription is measured.

In one embodiment, ubiquitination or degradation of c-fos, c-Jun, SMAD3, GATA3 or Runx2 is measured.

In one embodiment, AP-1, TRAF2 or Runx2 ubiquitination is measured.

In one embodiment, the binding of first and second polypeptide is inhibited.

In one embodiment, the binding of first and second polypeptide is stimulated.

In another aspect, the invention pertains to a method of identifying a compound that modulates a mammalian KRC biological activity comprising:

(a) contacting cells deficient in KRC or a molecule in a signaling pathway involving KRC with a test compound; and

(b) determining the effect of the test compound on the KRC biological activity, the test compound being identified as a modulator of the biological activity based on the ability of the test compound to modulate the biological activity in the cells deficient in KRC or a molecule in a signaling pathway involving KRC to thereby identify a compound that modulates a mammalian KRC biological activity.

In one embodiment, the biological activity of KRC is selected from the group consisting of modulation of: modulation of a TGFβ signaling pathway, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription, or modulation of osteocalcin gene transcription.

In one embodiment, the cells are in a non-human animal deficient in KRC or a molecule in a signal transduction pathway involving KRC and the cells are contacted with the test compound by administering the test compound to the animal.

In one aspect, the invention pertains to a method of identifying compounds useful in modulating a biological activity of mammalian KRC comprising, a) providing an indicator composition comprising mammalian KRC or a molecule in a signal transduction pathway involving KRC; b) contacting the indicator composition with each member of a library of test compounds; c) selecting from the library of test compounds a compound of interest that modulates a biological activity of KRC or the molecule in a signal transduction pathway involving KRC; to thereby identify a compound that modulates a biological activity of mammalian KRC, wherein the biological activity of KRC is selected from the group consisting of: modulation of a TGFβ signaling pathway, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription, and modulation of osteocalcin gene transcription.

In one embodiment, the indicator composition is a cell that expresses KRC, and at least one molecule selected from the group consisting of: c-Jun, c-Fos, AP-1, GATA3, SMAD, and Runx2 protein.

In one embodiment, the indicator composition is a cell free composition.

In one aspect, the invention pertains to a method for modulating the expression and/or biological activity of a KRC polypeptide in a subject, comprising contacting an immune cell from the subject with a compound that modulates the expression and/or biological activity of a KRC polypeptide in the immune cell, such that the expression and/or biological activity of the KRC polypeptide in the subject is modulated, wherein the biological activity of KRC is selected from the group consisting of: modulation of a TGFβ signaling pathway, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell

differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of Glα transcription, and modulation of osteocalcin gene transcription.

In one embodiment, the step of contacting occurs *in vivo*. In another embodiment, the step of contacting occurs *in vitro*.

In one embodiment, the cell is selected from the group consisting of: a T cell, a B cell, and a macrophage.

In one embodiment, KRC activity is enhanced. In another embodiment, KRC activity is inhibited.

In one embodiment, the agent is selected from the group consisting of: a nucleic acid molecule encoding a polypeptide comprising a biologically active KRC domain, a polypeptide comprising a biologically active KRC domain, and a small molecule KRC agonist.

In one embodiment, the agent is selected from the group consisting of: an intracellular antibody, a nucleic acid molecule that is antisense to a nucleic acid molecule encoding KRC, a KRC siRNA molecule, a dominant negative KRC molecule, and a small molecule KRC antagonist.

In another aspect, the invention pertains to a method for modulating the interaction between a KRC molecule and a KRC- binding partner comprising contacting an immune cell with an agent that modulates the interaction between KRC and a KRC-binding partner in the immune cell such that the interaction between KRC and a KRC-binding partner is modulated, wherein the KRC-binding partner is selected from the group consisting of c-Jun, GATA3, SMAD, or Runx2.

In one embodiment, the step of contacting occurs *in vivo*.

In another embodiment, the step of contacting occurs *in vitro*.

In one embodiment, the interaction between a KRC molecule and a KRC-binding partner molecule is inhibited.

In one embodiment, the agent is selected from the group consisting of: an intracellular antibody, a nucleic acid molecule that is antisense to a TRAF molecule, a nucleic acid molecule that is antisense to a c-Jun molecule, a nucleic acid molecule that is antisense to a KRC molecule, a nucleic acid molecule that is antisense to a c-Jun molecule a nucleic acid molecule that is antisense to a GATA3 molecule, a nucleic acid molecule that is antisense to a SMAD molecule, a nucleic acid molecule that is antisense

to a RUNX2 molecule, a dominant negative KRC molecule, a dominant negative c-Jun molecule, a dominant negative GATA3 molecule, a dominant negative SMAD molecule, and a dominant negative Runx2 molecule.

In one embodiment, the portion of KRC that interacts with c-Jun, GATA3, SMAD, or Runx2 comprises amino acid residues 204-1055 of KRC.

In one embodiment, the agent that modulates the interaction between a KRC molecule and a KRC- binding partner molecule is useful for the treatment of an autoimmune disease in a subject.

In one embodiment, the agent that modulates the interaction between a KRC molecule and a KRC- binding partner molecule is useful for the treatment of an malignancy in a subject.

In one embodiment, the agent that modulates the interaction between a KRC molecule and a KRC- binding partner molecule is useful for the treatment of a metabolic bone disease in a subject.

In one embodiment, the autoimmune disease is selected from the group consisting of: systemic lupus erythematosus; rheumatoid arthritis; goodpasture's syndrome; Grave's disease; Hashimoto's thyroiditis; pemphigus vulgaris; myasthenia gravis; scleroderma; autoimmune hemolytic anemia; autoimmune thrombocytopenic purpura; polymyositis and dermatomyositis; pernicious anemia; Sjögren's syndrome; ankylosing spondylitis; vasculitis, multiple sclerosis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, and type I diabetes mellitus.

In one embodiment, the malignancy is selected from the group consisting of: acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related lymphoma; B cell chronic lymphocytic leukemia; cancer of the bile duct; bladder cancer; bone cancer, osteosarcomal malignant fibrous histiocytomal brain stem gliomal brain tumor; breast cancer; bronchial adenomas; carcinoid tumors; adrenocortical carcinoma; central nervous system lymphoma; cancer of the sinus, cancer of the gall bladder; gastric cancer; cancer of the salivary glands; cancer of the esophagus; neural cell cancer; intestinal cancer (*e.g.*, of the large or small intestine); cervical cancer; colon cancer; colorectal cancer; cutaneous T-cell lymphoma; B-cell lymphoma; T-cell lymphoma; endometrial cancer; epithelial cancer; endometrial cancer; intraocular melanoma; retinoblastoma; hairy cell leukemia; liver cancer; Hodgkin's disease;

Kaposi's sarcoma; acute lymphoblastic leukemia; lung cancer; non-Hodgkin's lymphoma; melanoma; multiple myeloma; neuroblastoma; prostate cancer; retinoblastoma; Ewing's sarcoma; vaginal cancer; Waldenstrom's macroglobulinemia; adenocarcinomas; ovarian cancer, chronic lymphocytic leukemia, pancreatic cancer; and Wilm's tumor.

In one embodiment, the metabolic bone disease is selected from the group consisting of: osteoporosis, osteomalacia, skeletal changes of hyperparathyroidism and chronic renal failure (renal osteodystrophy) and osteitis deformans (Paget's disease of bone).

In another aspect, the invention pertains to a method for inhibiting a neoplasia in a subject, comprising contacting a tumor cell from the subject with a compound that modulates the expression and/or biological activity of KRC in the tumor cell such that the neoplasia in the subject is inhibited.

In one embodiment, the neoplasia is a B cell chronic lymphocytic leukemia

In another embodiment, the invention pertains to a non-human animal, in which the gene encoding the KRC gene is misexpressed.

In one embodiment, the animal is a transgenic animal.

In one embodiment, the transgenic animal is a mouse.

In one embodiment, the KRC gene is disrupted by removal of DNA encoding all or part of the KRC protein.

In one embodiment, the animal is homozygous for the disrupted gene.

In one embodiment, the animal is heterozygous for the disrupted gene.

In one embodiment, the animal is a transgenic mouse with a transgenic disruption of the KRC gene.

In one embodiment, the disruption is an insertion or deletion.

In one aspect, the invention pertains to a transgenic mouse comprising in its genome an exogenous DNA molecule that functionally disrupts a KRC gene of said mouse, wherein said mouse exhibits a phenotype characterized by impaired Th2 cell development, decreased Th2 cytokine production, impaired TGFβR signaling in B cells, decreased IgA secretion and decreased transcription of the GLα gene, relative to a

wildtype mouse.

Brief Description of the Figures

Figure 1(A) – 1(E) show the interaction of amino acid residues 204 to 1055 of KRC ("KRC tr") (amino acids 204-1055 of SEQ ID NO:2) with TRAF family members. *Figure 1(A)* shows a schema of KRC constructs used. *Figure 1(B)* upper panel depicts the interaction of KRC tr with TRAFs in mammalian cells. 293 T cells were cotransfected with the indicated FLAG-TRAFs and MYC-tagged KRC tr, and immunoprecipitated with anti-MYC antibody, followed by blotting with anti-FLAG antibody. *Figure 1(B) l*ower panel depicts the direct western blot of overexpressed TRAFS and KRC tr with anti-FLAG or anti-MYC. *Figure 1(C)* depicts the differential interaction of KRC tr with TRAF proteins. The coimmunoprecipitation experiments were performed in the presence of 300 mM NaCl instead of 137 mM NaCl. *Figure 1(D)* depicts KRC tr interacting with TRAF2 lacking the Ring finger domain. 293 T cells were transfected with MYC-KRC tr and with FLAG tagged TRAF2 or with FLAG-tagged TRAF2 (87-501). *Figure 1(E)* depicts the interaction of KRC tr with endogenous TRAF2 but not with endogenous TRAF5 or TRAF6. 293T were transfected with an expression vector encoding an MYC-tagged KRC tr, or empty plasmid. Lysates from 293T cells were incubated with anti-MYC antibodies. Coimmunoprecipitated endogenous TRAF was detected by western blotting with specific anti-TRAF antibodies.

Figure 2(A) –2(C) depicts KRC preventing TRAF-dependent NFkB activation. Inhibition of TRAF2 (*Figure 2(A)*), TRAF5 (*Figure 2(B)*) and TRAF6 (*Figure 2(C)*) mediated activation of NFkB by ectopically expressed KRC. 293 T cells (3 X10^5) were transfected with 25 ng of NFkB luciferase reporter plasmid, 50 ng of CMVβGal and 1 μg of each indicated plasmid and 24 hours post transfection cells were harvested. Data from at least five experiments normalized for β galactosidase activity are shown. Vec refers to the empty MYC vector without the addition of TRAFs.

Figures 3A-3C shows that KRC and KRC tr inhibit while antisense and dominant negative KRC increase TNFα-driven NFκB transactivation. 293 T cells (3 X10^5) were transfected with 25 ng of NFκB luciferase reporter plasmid, 50 ng of CMVβGal and 1 μg of each indicated plasmid and 24 hours post transfection cells were

stimulated for 4 hours with 10 ng/ml of TNFα. (A) KRC and KRC tr (B) dominant negative and antisense KRC (C) antisense KRC in the presence of exogenous TRAF2. Data from at least five experiments normalized for β galactosidase activity are shown.

Figure 4 shows that IKKβ (IκB kinase) overexpression overcomes KRC inhibition of NFκB-dependent transactivation. 293 T cells (3 $X10^5$) were transfected with 25 ng of NFκB luciferase reporter plasmid, with 50 ng of CMV βGal, 200 ng of IKKβ expression vector when indicated and 1 μg of each indicated plasmid and cells harvested 24 hours post transfection. Data from two experiments normalized for β galactosidase activity are shown.

Figure 5 shows that KRC increases TNFα–induced apoptosis. 3T3 cells were cotransfected with CMV lacZ vector (300 ng per plate) and either empty expression vector or the expression vectors indicated (2μg of each). Half of the transfected cultured cells were treated with TNFα (20 ng/ml) at 12 hours after the transfection and the other half left untreated. All the cells were fixed and stained at 36 hours after the transfection. The number of blue cells in each transfection was determined by counting six different fields. A representative experiment of three performed is presented.

Figures 6A-C show that KRC prevents TRAF2 and TNFα-dependent JNK activation. Inhibition of TRAF2 (A) and TNFα (B, C) mediated JNK/SAPK activation by ectopic expression of KRC. (A) 293 T cells were transfected with 400 ng of TRAF2 and 2 μg of the indicated expression vector. Twenty-four hours after the transfection, the cells were harvested and lysed, and the endogenous JNK was precipitated with 5 μg of GST-cJUN (1-79) for 4 hours. JNK activity was determined by using GST-cJUN (1-79) as a substrate. (B,C) 293 T cells were cotransfected with vectors encoding HA-tagged JNK2 (500ng) and the indicated expression vector (2 μg). Twenty-four hours after the transfection cells were stimulated for 10 min with 10 ng/ml of TNFα and cells harvested at varying time points. JNK activity was assayed with GST-cJUN (1-79) as substrate.

Figures 7A-B show that KRC is a negative regulator of endogenous TNFα expression. Northern blotting analysis was performed using total RNA made from RAW cell lines transfected with an empty vector as a control and from a panel of 9 independent RAW clones stably transfected with full-length KRC (upper) and 3 RAW

clones stably transfected with dominant negative KRC (lower). The blot was probed with a TNFα cDNA and with HPRT as loading control.

Figure 8 shows that KRC is present in both cytosol and nucleus. GFP-tagged KRC was stably transfected into NIH 3T3 cells, and cells examined by fluorescence microscopy immediately after trypsinization (left panel) or after adherence to glass slides (right panel).

Figure 9 shows that KRC is Th1-specific. RT-PCR analysis of KRC expression in primary T cells was performed. KRC expression was measured at 24 hours and 72 hours. The results demonstrate that KRC expression is rapidly lost in Th2 cells at 72 hours whereas KRC expression in Th1 cells is maintained at 72 hours.

Figures 10A-D shows that KRC activates T cells. KRC was transfected into Jurkat T cells and CD69 expression was measured by FACS analysis. The results show that KRC overexpression increases CD69 expression in Jurkat T cells.

Figures 11(A)-11(C) show that KRC increases IL-2 gene transcription in the presence of PMA/Ionomycin and does so primarily through activating AP-1 with no contribution from NFAT. *Figure 11(A)* shows IL-2 promoter transactivation by KRC in Jurkat T cells activated by PMA/Ionomycin. *Figure 11(B)* shows transactivation of a composite NFAT-AP1 reporter by KRC. *Figure 11(C)* shows transactivation of an AP-1 reporter by KRC.

Figures 12(A)-12(C) show that KRC increases IL-2 gene transcription in the presence of B cell antigen presenting cells and superantigen SEE and does so primarily through activating AP-1 with no contribution from NFAT. *Figure 12(A)* shows IL-2 promoter transactivation by KRC in Jurkat T cells activated by the Raji B cell APC line and the superantigen SEE. *Figure 12(B)* shows transactivation of a composite NFAT-AP1 reporter by KRC. *Figure 12(C)* shows transactivation of an AP-1 reporter by KRC.

Figures 13(A)-13(B) show that KRC IL-2 production. IL-2 production was measured by ELISA. *Figure 13(A)* shows stable transfectants and *Figure 13(B)* shows CD3 and CD3+CD28 stimulated cells.

Figures 14(A)-14(B) show that KRC transactivation of AP-1 response element depends on Ras, Raf and PKC-theta signaling molecules. *Figure 14(A)* shows KRC transactivation of the AP-1 reporter is blocked by dominant negative Ras and Raf.

Figure 14(B) shows KRC transactivation of the AP-1 reporter is blocked by dominant negative PKC-theta and by the specific PKC-theta inhibitor Rottlerin.

Figure 15 shows that KRC controls IL-2 expression. RT-PCR of KRC transfected Jurkat clones was performed. The results show increased IL-2 expression.

Figure 16 shows that KRC increases actin polymerization. Immunofluorescence of F-actin upon KRC overexpression in Jurkat T cells was performed. The results show the reorganization of F-actin filaments in KRC transfected Jurkat T cells.

Figure 17 shows that KRC expression increases in CD4 cells upon activation. Primary C57/B6 CD4+ T cells were stimulated with anti-CD3 (2.0 μg/mL)/anti-CD28 (1.0 μg/mL) antibodies for the indicated times. RNA was prepared and KRC expression was determined by RT-PCR, with ß-actin as an internal control.

Figures 18A-D show that KRC overexpression increases while KRC loss decreases endogenous IL-2 production. In panel A Jurkat T cells were stably transfected with vector (pEF) or KRC expression plasmids. Stable clones were stimulated for 18 hours with PMA (50 ng/mL) plus ionomycin (2 μM) and IL-2 production was measured by ELISA. In panel B Primary CD4+ T cells were activated for 36 hours and subsequently transduced with control (RV), KRC, or KRC dominant negative (ZAS2) bicistronic GFP-expressing retroviruses. GFP-positive cells were sorted and stimulated for 24 hours with anti-CD3 or anti-CD3/anti-CD28 antibodies and IL-2 production was measured by ELISA. In panel C CD4 T cells from KRC +/+ or -/- mice were stimulated with anti-CD3 (1.0 μg/mL)/CD28 (0.5 μg/mL) antibodies for 24 hours and IL-2 production was measured by ELISA. In panel D CD4 T cells from KRC +/+ or -/- mice were stimulated with anti-CD3/CD28 antibodies for 72 hours in the presence of 200 U/mL human IL-2. IFNγ production was measured by ELISA.

Figures 19(A)-19(C) show that KRC overexpression increases the transcription of the IL-2 gene. *Figure 19 (A)* Stably transfected Jurkat T cell clones with vector (vec) or KRC (Jurkat-KRC) were stimulated with PMA (50 ng/mL) plus ionomycin (2 μM) for 6 hours. IL-2 mRNA abundance was determined by RT-PCR with tubulin as an internal control. *Figure 19 (B)* Jurkat cells were transiently transfected with an IL-2-Luciferase reporter along with Vector, KRC, or KRCtr (amino acids 204-1055) and, in all cases, a CMV-ß-Gal reporter as an internal control (see text for details). 24

hours later, cells were stimulated with PMA plus ionomycin for 6 hours (upper panel) or Raji cells loaded with SEE for 8 hours (lower panel). Luciferase activity was determined and normalized for ß-Galactosidase activity. *Figure 19 (C)* Jurkat cells were transiently transfected with NFAT/AP-1-, NFAT-, or AP-1-Luciferase reporters and treated as above.

Figures 20(A)-20(C) show that KRC does not modulate MAPK activity. *Figure 20 (A)* Jurkat cells were transiently transfected with AP-1 Luciferase reporter along with KRC and RasN17 DN vectors. 24 hours later cells were pretreated with Rottlerin (10 μM) and stimulated for 6 hours with PMA plus ionomycin. Luciferase activity was measured as above. *Figure 20 (B)* Jurkat cells were transfected with a GAL4 Luciferase reporter along with a GAL4 DNA binding domain, GAL4-ATF2, or GAL4-ELK1 with or without KRC. 24 hours later, cells were stimulated with PMA plus ionomycin and analyzed for Luciferase activity as above. *Figure 20 (C)* Jurkat cells were transiently transfected with FLAG-JNK2, and either Vector, KRC, or MKK7. 48 hours later, cells were stimulated with PMA plus ionomycin for 6 hours and JNK activity was determined by immunoprecipitation/kinase assay. Equal amounts of FLAG-Jnk2 protein were immunoprecipitated, as judged by anti-FLAG western blot (lower panel).

Figures 21(A)-21(D) show that KRC physically interacts with c-Jun and acts as a transcriptional coactivator. *Figure 21 (A)* 293T cells were transfected with c-Jun and myc-KRCtr. 48 hours later, lysates were immunoprecipitated with anti-Myc antibody. Immunoprecipitates were probed by western blotting with anti-c-Jun antibody. *Figure 21 (B)* (left panel) 293T cells were cotransfected with c-Jun and full length His-KRC. 48 hours later, lysates were immunoprecipitated with anti-His antibody (DE8 Omniprobe) and precipitates were probed by western blotting with anti-c-Jun antibody. (right panel) *In vitro* translated and S35-labelled c-Jun and His-KRCtr were mixed and immunoprecipitated with anti-His antibody. Recovered c-Jun protein was visualized by autoradiography. *Figure 21 (C)* Jurkat or EL4 T cells were stimulated with PMA plus ionomycin for 45 minutes. Lysates were immunoprecipitated with anti-c-Jun antibody, and immunoprecipitates were probed with specific anti-KRC rabbit antisera. *Figure 21 (D)* (upper panel) 293T cells were transfected with AP-1 Luciferase along with c-Jun, c-Fos, and KRC. 24 hours later, Luciferase activity was determined as above. (lower panel) 293T cells were transfected with GAL4 Luciferase along with GAL4, GAL4-c-

Jun 1-224, or GAL4-c-Fos 208-313. 24 hours later, luciferase activity was determined as above.

Figures 22(A)-22(D) show that KRC regulates the stability of the c-Jun/c-Fos AP-1 transcription factor by controlling its degradation. *Figure 22(A)* shows that the stability of the c-Fos protein in the presence of cycloheximide was compromised in the presence of KRC and dramatically stabilized in the presence of the KRC dominant negative expressing only the ZAS2 domain or in the presence of the antisense KRC. *Figure 22(B)*, shows that overexpression of antisense KRC, by inhibiting the expression of endogenous KRC, decreased the rate of c-Jun degradation. *Figure 22(C)*, show that overexpression of full-length KRC, in the presence of low dose cycloheximide blocked endogeneous protein synthesis and led to the rapid degradation of c-Jun. *Figure 22(D)* shows the specificity of KRC for the c-Jun/c-Fos AP-1 pair since KRC was unable to promote the degradation of other fos family members Fra1, Fra2 and Fos B.

Figures 23A-23F show that KRC interacts with and augments the transcriptional activity of Smad3. (23A) Decreased serum IgA levels in non-immunized KRC -/- mice. (23B) Marked reduction in the levels of in vitro IgA secretion and (23C) IgCα GLT expression by KRC -/- B cells. (23D) Co-expression of KRC enhances the transcriptional activity of Runyx3 and Smad3 to induces expression of the mouse GLα promoter {-1797+46) luciferase reporter plasmid. (23E) KRC physically interacts with Smad3 and to a lesser extent Smad2 but docs not interact with Smad1 or Smad4. (23F) KRC augments the transcriptional activity of Smad3 to induce expression of a SBE-luciferase construct.

Figures 24A-24J show that KRC augments Th2 cytokine production and interacts with GATA3.

Figure 25 shows KRC degrades its partners, c-Jun, c-fos, SMAD3, Runx2, GATA3, and TRAF2.

Figure 26 shows that KRC ubiquitinates TRAF2 and Runx2.

Figure 27 shows that Shn2 and KRC associate with AP-1 to transactivate an AP-1 reporter.

Detailed Description of the Invention

The present invention is based, at least in part, on the discovery that KRC molecules regulate a wide variety of cellular processes, including inhibiting NFkB transactivation, increasing TNF-alpha induced apoptosis, inhibiting JNK activation, inhibiting endogenous TNF-alpha expression, activating immune cell proliferation and immune cell activation (*e.g.*, in Th1 cells), activating IL-2 expression *e.g.*, by activating the AP-1 transcription factor, and increasing actin polymerization.

The present invention also demonstrates that that KRC interacts with TRAF molecules. The interaction between KRC and TRAF involves the C domain of TRAF and amino acid residues 204 to 1055 of KRC. Furthermore, the present invention demonstrates that KRC physically interacts with the c-Jun component of AP-1 to control its degradation. KRC also interacts with GATA3, SMAD, *e.g.*, SMAD2 and SMAD3, and Runx2 to control their degradation, and ubiquitinates TRAF and Runx2.

Furthermore, the present invention demonstrates upon TCR stimulation KRC also associates with the Th2-specific transcription factor GATA3, and T cells lacking KRC have impaired production of GATA3 dependent Th2 cytokines, such as, IL-4, IL-5 and IL-13. In addtion, upon TGFβ receptor signaling, KRC physically associates with members of the SMAD transcription factor family, *e.g.*, SMAD2 and SMAD3, to activate IgA germline transcription in B cells.

The KRC protein (for κB binding and putative recognition component of the V(D)J Rss), referred to interchangeably herein as *Schnurri-3* (Shn3), is a DNA binding protein comprised of 2282 amino acids. KRC has been found to be present in T cells, B cells, and macrophages. The KRC cDNA sequence is set forth in SEQ ID NO:1. The amino acid sequence of KRC is set forth in SEQ ID NO:2. KRC is a member of a family of zinc finger proteins that bind to the kB motif (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648). Zinc finger proteins are divided into three classes represented by KRC and the two MHC Class I gene enhancer binding proteins, MBP1 and MBP2 (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648).
Zinc finger proteins are identified by the presence of highly conserved Cys2His2 zinc fingers. The zinc fingers are an integral part of the DNA binding structure called the ZAS domain. The ZAS domain is comprised of a pair of zinc fingers, a glutamic acid/aspartic acid-rich acidic sequence and a serine/threonine rich sequence. The ZAS

domains have been shown to interact with the kB like *cis*-acting regulatory elements found in the promoter or enhancer regions of genes. The genes targeted by these zinc finger proteins are mainly involved in immune responses.

The KRC ZAS domain, in particular, has a pair of Cys2-His2 zinc fingers followed by a glutamic acid/aspartic acid-rich acidic sequence and five copies of the serine/threonine-proline-X-arginine/lysine sequence. Southwestern blotting experiments, electrophoretic mobility shift assays (EMSA) and methylation interference analysis has also demonstrated that KRC recombinant proteins bind to the κB motif as well as to the Rss sequence (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001) and do so in highly ordered complexes (Mak, C. H., *et al.* 1994. *Nuc.Acid Res. 22*, 383-390.; Wu *et al.* 1998. *Science 281*, 998-1001).

Similar zinc finger-acidic domain structures are present in human KBP1, MBP1 and MBP2, rat ATBP1 and ATBP2, and mouse αA-CRYBP proteins. KRC has recently been shown to regulate transcription of the mouse metastasis-associated gene, *s100A4/mts1**, by binding to the Sb element (a kB like sequence) of the gene. (Hjelmsoe, I., *et al.* 2000. *J. Biol. Chem.* 275(2): 913-920). KRC is regulated by post-translational modification as evidenced by the fact that pre-B cell nuclear protein kinases phosphorylate KRC proteins on serine and tyrosine residues. Phosphorylation increases DNA binding, providing a mechanism by which KRC may respond to signals transmitted from the cell surface (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648). Two prominent ser/thr-specific protein kinases that play a central role in signal transduction are cyclic AMP-dependent protein kinase A (PKA) and the protein kinase C (PKC family). Numerous other serine/threonine specific kinases, including the family of mitogen-activated protein (MAP) kinases serve as important signal transduction proteins which are activated in either growth-factor receptor or cytokine receptor signaling. Other protein ser/thr kinases important for intracellular signaling are Calcium-dependent protein kinase (CaM-kinase II) and the c-raf-protooncogene. KRC is known to be a substrate for epidermal growth factor receptor kinase and p34cdc2 kinase *in vitro*.

The results of a yeast two hybrid screen using amino acid residues 204 to 1055 of KRC (which includes the third zinc finger) as bait demonstrate that KRC interacts with the TRAF family of proteins and that this interaction occurs through the

TRAF C domain and that KRC interacts with higher affinity with TRAF2 than with TRAF5 and TRAF6. (See Example 1).

Recent research has lead to the isolation of polypeptide factors named TRAFs for tumor necrosis factor receptor associated factors, which participate in the TNFR signal transduction cascade. Six members of the TRAF family of proteins have been identified in mammalian cells (reviewed in Arch, R.H., *et al.* 1998. *Genes Dev. 12*, 2821-2830). All TRAF proteins, with the exception of TRAF1, contain an amino terminal RING finger domain with a characteristic pattern of cysteines and histidines that coordinate the binding of Zn2+ ions (Borden, K. L. B., *et al.* 1995. *EMBO J 14*, 1532-1521), which is followed by a stretch of multiple zinc fingers. All TRAFs share a highly conserved carboxy-terminal domain (TRAF-C domain) which is required for receptor binding and can be divided into two parts, a highly conserved domain which mediates homo and heterodimerization of TRAF proteins and also the association of the adapter proteins with their associated receptors and an amino-terminal half that displays a coiled-coil configuration. TRAF molecules have distinct patterns of tissue distribution, are recruited by different cell surface receptors and have distinct functions as revealed most clearly by the analysis of TRAF-deficient mice (*see* Lomaga, M. A., *et al.* 1999. *Genes Dev. 13*, 1015-24; Nakano, H., *et al.* 1999. *Proc. Natl. Acad. Sci. USA 96*, 9803-9808; Nguyen, L. T., *et al.* 1999. *Immunity 11*, 379-389; Xu, Y., *et al.* 1996. *Immunity 5*, 407-415.; Yeh, W. C., *et al.* 1997. *Immunity 7*, 715-725).

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages which elicits a wide range of biological effects. These include an important role in endotoxic shock and in inflammatory, immunoregulatory, proliferative, cytotoxic, and anti-viral activities (reviewed by Goeddel, D. V. *et al.*, 1986. *Cold Spring Harbor Symposia on Quantitative Biology* 51: 597-609; Beutler, B. and Cerami, A., 1988. *Ann. Rev. Biochem.* 57: 505-518; Old, L. J., 1988. *Sci. Am.* 258(5): 59-75; Fiers, W. 1999. *FEBS Lett.* 285(2):199-212). The induction of the various cellular responses mediated by TNF is initiated by its interaction with two distinct cell surface receptors, an approximately 55 kDa receptor termed TNFR1 and an approximately 75 kDa receptor termed TNFR2. Human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. *et al.*, 1990. *Cell* 61:351; Schall, T. J. *et al.*, 1990. *Cell* 61: 361; Smith, C. A. *et al.*, 1990 *Science* 248: 1019; Lewis, M. *et al.*,

1991. *Proc. Natl. Acad. Sci. USA* 88: 2830-2834; Goodwin, R. G. *et al.*, 1991. *Mol. Cell. Biol.* 11:3020-3026).

TNFα binds to two distinct receptors, TNFR1 and TNFR2, but in most cell types NFκB activation and JNK/SAPK activation occur primarily through TNFR1. TNFR1 is known to interact with TRADD which functions as an adaptor protein for the recruitment of other proteins including RIP, a serine threonine kinase, and TRAF2. Of the six known TRAFs, TRAF2, TRAF5 and TRAF6 have all been linked to NFκB activation (Cao, Z., *et al.* 1996. *Nature 383:* 443-6; Rothe, M., *et al.* 1994. *Cell 78:* 681-692; Nakano, H., *et al.* 1996. *J. Biol. Chem. 271*:14661-14664), and TRAF2 in particular has been linked to activation of the JNK/SAPK proteins as shown unequivocally by the failure of TNFα to activate this MAP kinase in cells lacking TRAF2 or expressing a dominant negative form of TRAF2 (Yeh, W. C., *et al.* 1997. *Immunity 7*: 715-725; Lee, S. Y., *et al.* 1997. *Immunity 7:*1-20).

Various aspects of the invention are described in further detail in the following subsections:

I. Definitions

As used herein, the term "KRC" , used interchangeably with "Shn3" refers to κB binding and putative recognition component of the V(D)I Rss. The nucleotide sequence of KRC is set forth in SEQ ID NO:1 and the amino acid sequence of KRC is set forth in SEQ ID NO:2. The amino acid sequence of the ZAS domain of KRC is set forth in amino acids 1497-2282 of SEQ ID NO:2 (SEQ ID NO:8). The amino acid sequence of KRC tr is shown in amino acid residues 204 to 1055 of SEQ ID NO:2. As used herein, the term "KRC", unless specifically used to refer a specific SEQ ID NO, will be understood to refer to a KRC family polypeptide as defined below.

"KRC family polypeptide" is intended to include proteins or nucleic acid molecules having a KRC structural domain or motif and having sufficient amino acid or nucleotide sequence identity with a KRC molecule as defined herein. Such family members can be naturally or non-naturally occurring and can be from the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or, alternatively, can contain homologues

of non-human origin. Preferred members of a family may also have common functional characteristics. Preferred KRC polypeptides comprise one or more of the following KRC characteristics: a pair of Cys2-His2 zinc fingers followed by a Glu- and Asp-rich acidic domain and five copies of the ser/Thr-Pro-X-Arg/Lys sequence thought to bind DNA.

As used herein, the term "KRC activity", "KRC biological activity" or "activity of a KRC polypeptide" includes the ability to modulate an activity regulated by KRC or a signal transduction pathway involving KRC. For example, in one embodiment a KRC biological activity includes modulation of an immune response. Exemplary KRC activities include *e.g.*, modulating: immune cell activation and/or proliferation (such as by modulating cytokine gene expression), cell survival (*e.g.*, by modulating apoptosis), signal transduction via a signaling pathway (*e.g.*, an NFkB signaling pathway, a JNK signaling pathway, and/or a TGFβ signaling pathway), actin polymerization, ubiquitination of AP-1, ubiquitination of TRAF, ubiquitination of Runx2, degradation of c-Jun, degradation of c-Fos, degradation of SMAD, degradation of Runx 2, degradation of GATA3, GATA3 expression, Th2 cell differentiation, Th2 cytokine production, IgA production, GLα transcription, and/or osteocalcin gene transcription.

As used herein, the various forms of the term "modulate" are intended to include stimulation (*e.g.*, increasing or upregulating a particular response or activity) and inhibition (*e.g.*, decreasing or downregulating a particular response or activity).

As described in the appended Examples, KRC increases immune cell activation and cytokine production. In addition, when KRC is overexpressed, it results in the inhibition of NFkB and JNK signaling pathways. Inhibition of these pathways is associated with cellular inflammatory and apoptotic responses. In one embodiment, the KRC activity is a direct activity, such as an association with a KRC-target molecule or binding partner. As used herein, a "target molecule", "binding partner" or "KRC binding partner" is a molecule with which a KRC protein binds or interacts in nature, such that KRC mediated function is achieved.

As used herein the term "TRAF" refers to TNF Receptor Associated Factor (See *e.g.*, Wajant et al, 1999, *Cytokine Growth Factor Rev* 10:15-26). The "TRAF" family includes a family of cytoplasmic adapter proteins that mediate signal transduction from many members of the TNF-receptor superfamily and the interleukin-1

receptor (see *e.g.*, Arch, R.H. *et al.*, 1998, *Genes Dev.* 12:2821-2830). As used herein, the term "TRAF C domain" refers to the highly conserved sequence motif found in TRAF family members.

As used herein, the terms "TRAF interacting portion of a KRC molecule" or "c-Jun interacting portion of a KRC molecule" includes a region of KRC that interacts with TRAF or c-Jun. In a preferred embodiment, a region of KRC that interacts with TRAF or c-Jun is amino acid residues 204-1055 of SEQ ID NO:2 (SEQ ID NO:7). As used herein, the term "KRC interacting portion of a TRAF molecule" or "KRC interacting portion of a TRAF molecule" includes a region of TRAF or c-Jun that interacts with KRC. In a preferred embodiment, a region of TRAF that interacts with KRC is the TRAF C domain.

The term "interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a yeast two hybrid assay or coimmunoprecipitation. The term interact is also meant to include "binding" interactions between molecules. Interactions may be protein-protein or protein-nucleic acid in nature.

As used herein, the term "contacting" (*i.e.*, contacting a cell *e.g.* an immune cell, with an compound) is intended to include incubating the compound and the cell together *in vitro* (*e.g.*, adding the compound to cells in culture) or administering the compound to a subject such that the compound and cells of the subject are contacted *in vivo*. The term "contacting" is not intended to include exposure of cells to a KRC modulator that may occur naturally in a subject (*i.e.*, exposure that may occur as a result of a natural physiological process).

As used herein, the term "test compound" includes a compound that has not previously been identified as, or recognized to be, a modulator of KRC activity and/or expression and/or a modulator of cell growth, survival, differentiation and/or migration.

The term "library of test compounds" is intended to refer to a panel comprising a multiplicity of test compounds.

As used herein, the term "cell free composition" refers to an isolated composition which does not contain intact cells. Examples of cell free compositions include cell extracts and compositions containing isolated proteins.

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

In one embodiment, nucleic acid molecule of the invention is an siRNA molecule. In one embodiment, a nucleic acid molecule of the invention mediates RNAi. RNA interference (RNAi) is a post-transcriptional, targeted gene-silencing technique that uses double-stranded RNA (dsRNA) to degrade messenger RNA (mRNA) containing the same sequence as the dsRNA (Sharp, P.A. and Zamore, P.D. 287, 2431-2432 (2000); Zamore, P.D., *et al. Cell* 101, 25-33 (2000). Tuschl, T. *et al. Genes Dev.* 13, 3191-3197 (1999); Cottrell TR, and Doering TL. 2003. Trends Microbiol. 11:37-43; Bushman F.2003. Mol Therapy. 7:9-10; McManus MT and Sharp PA. 2002. Nat Rev Genet. 3:737-47). The process occurs when an endogenous ribonuclease cleaves the longer dsRNA into shorter, e.g., 21- or 22-nucleotide-long RNAs, termed small interfering RNAs or siRNAs. The smaller RNA segments then mediate the degradation of the target mRNA. Kits for synthesis of RNAi are commercially available from, *e.g.* New England Biolabsor Ambion. In one embodiment one or more of the chemistries described above for use in antisense RNA can be employed in molecules that mediate RNAi.

As used herein, the term "immune response" includes immune cell-mediated (*e.g.*, T cell and/or B cell-mediated) immune responses that are influenced by modulation of immune cell activation. Exemplary immune responses include B cell responses (*e.g.*, antibody production, *e.g.*, IgA production), T cell responses (*e.g.*, proliferation, cytokine production and cellular cytotoxicity), and activation of cytokine responsive cells, *e.g.*, macrophages. In one embodiment of the invention, an immune response is T cell mediated. In another embodiment of the invention, an immune response is B cell mediated. As used herein, the term "downregulation" with reference to the immune response includes a diminution in any one or more immune responses, preferably T cell responses, while the term "upregulation" with reference to the immune response includes an increase in any one or more immune responses, preferably T cell responses. It will be understood that upregulation of one type of immune response may

lead to a corresponding downregulation in another type of immune response. For example, upregulation of the production of certain cytokines (*e.g.*, IL-10) can lead to downregulation of cellular immune responses. In addition, it will be understood that KRC may have one effect on immune responses in the context of T cell receptor-mediated signaling, another in the context of TNFα-mediated signaling, and another in the context of TGFβ-mediated signaling.

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response. immune cells include lymphocytes, such as B cells and T cells; natural killer cells; and myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

The terms "antigen presenting cell" and "APC", as used interchangeably herein, include professional antigen presenting cells (*e.g.*, B lymphocytes, monocytes, dendritic cells, and Langerhans cells) as well as other antigen presenting cells (*e.g.*, keratinocytes, endothelial cells, astrocytes, fibroblasts, and oligodendrocytes).

As used herein, the term "T cell" (*i.e.*, T lymphocyte) is intended to include all cells within the T cell lineage, including thymocytes, immature T cells, mature T cells and the like, from a mammal (*e.g.*, human). T cells include mature T cells that express either CD4 or CD8, but not both, and a T cell receptor. The various T cell populations described herein can be defined based on their cytokine profiles and their function.

As used herein "progenitor T cells" ("Thp") are pluripotent cells that express both CD4 and CD8.

As used herein, the term "naïve T cells" includes T cells that have not been exposed to cognate antigen and so are not activated or memory cells. Naïve T cells are not cycling and human naïve T cells are CD45RA+. If naïve T cells recognize antigen and receive additional signals depending upon but not limited to the amount of antigen, route of administration and timing of administration, they may proliferate and differentiate into various subsets of T cells, *e.g.*, effector T cells.

As used herein, the term "differentiated" refers to T cells that have been contacted with a stimulating agent and includes effector T cells (e.g., Th1, Th2) and memory T cells. Differentiated T cells differ in expression of several surface proteins compared to naïve T cells and secrete cytokines that activate other cells.

As used herein, the term "memory T cell" includes lymphocytes which, after exposure to antigen, become functionally quiescent and which are capable of surviving for long periods in the absence of antigen. Human memory T cells are CD45RA-.

As used herein, the term "effector T cell" includes T cells which function to eliminate antigen (*e.g.*, by producing cytokines which modulate the activation of other cells or by cytotoxic activity). The term "effector T cell" includes T helper cells (*e.g.*, Th1 and Th2 cells) and cytotoxic T cells. Th1 cells mediate delayed type hypersensitivity responses and macrophage activation while Th2 cells provide help to B cells and are critical in the allergic response (Mosmann and Coffman, 1989, *Annu. Rev. Immunol.* 7, 145-173; Paul and Seder, 1994, *Cell* 76, 241-251; Arthur and Mason, 1986, *J. Exp. Med.* 163, 774-786; Paliard *et al.*, 1988, *J. Immunol.* 141, 849-855; Finkelman *et al.*, 1988, *J. Immunol.* 141, 2335-2341). As used herein, the term " T helper type 1 response" (Th1 response) refers to a response that is characterized by the production of one or more cytokines selected from IFN-γ, IL-2, TNF, and lymphotoxin (LT) and other cytokines produced preferentially or exclusively by Th1 cells rather than by Th2 cells. As used herein, a "T helper type 2 response" (Th2 response) refers to a response by $CD4^+$ T cells that is characterized by the production of one or more cytokines selected from IL-4, IL-5, IL-6 and IL-10, and that is associated with efficient B cell "help" provided by the Th2 cells (*e.g.*, enhanced IgG1 and/or IgE production).

As used herein, the term "regulatory T cell" includes T cells which produce low levels of IL-2, IL-4, IL-5, and IL-12. Regulatory T cells produce TNFα, TGFβ, IFN-γ, and IL-10, albeit at lower levels than effector T cells. Although TGFβ is the predominant cytokine produced by regulatory T cells, the cytokine is produced at lower levels than in Th1 or Th2 cells, *e.g.*, an order of magnitude less than in Th1 or Th2 cells. Regulatory T cells can be found in the CD4+CD25+ population of cells (see, *e.g.*, Waldmann and Cobbold. 2001. *Immunity*. 14:399). Regulatory T cells actively suppress the proliferation and cytokine production of Th1, Th2, or naïve T cells which have been stimulated in culture with an activating signal (*e.g.*, antigen and antigen presenting cells or with a signal that mimics antigen in the context of MHC, *e.g.*, anti-CD3 antibody plus anti-CD28 antibody).

As used herein, the term "receptor" includes immune cell receptors that bind antigen, complexed antigen (*e.g.*, in the context of MHC molecules), or antibodies. Activating receptors include T cell receptors (TCRs), B cell receptors (BCRs), cytokine receptors, LPS receptors, complement receptors, and Fc receptors. For example, T cell receptors are present on T cells and are associated with CD3 molecules. T cell receptors are stimulated by antigen in the context of MHC molecules (as well as by polyclonal T cell activating reagents). T cell activation via the TCR results in numerous changes, *e.g.*, protein phosphorylation, membrane lipid changes, ion fluxes, cyclic nucleotide alterations, RNA transcription changes, protein synthesis changes, and cell volume changes.

As used herein, the term "dominant negative" includes KRC molecules (*e.g.*, portions or variants thereof) that compete with native (*i.e.*, wild-type) KRC molecules, but which do not have KRC activity. Such molecules effectively decrease KRC activity in a cell.

As used herein, the term "inflammation" includes a response to injury which results in a dilation of the blood capillaries, a decrease in blood flow and an accumulation of leucocytes at the site of injury.

As used herein the term "apoptosis" includes programmed cell death which can be characterized using techniques which are known in the art. Apoptotic cell death can be characterized, *e.g.*, by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage. As used herein, the term "modulating apoptosis" includes modulating programmed cell death in a cell, such as a epithelial cell. As used herein, the term "modulates apoptosis" includes either up regulation or down regulation of apoptosis in a cell. Modulation of apoptosis is discussed in more detail below and can be useful in ameliorating various disorders, *e.g.*, neurological disorders.

As used herein, the term "NFkB signaling pathway" refers to any one of the signaling pathways known in the art which involve activation or deactivation of the transcription factor NFkB, and which are at least partially mediated by the NFkB factor (Karin, 1998, *Cancer J from Scientific American*, 4:92-99; Wallach et al, 1999, *Ann Rev of Immunology*, 17:331-367). Generally, NFkB signaling pathways are responsive to a

number of extracellular influences *e.g.* mitogens, cytokines, stress, and the like. The NFkB signaling pathways involve a range of cellular processes, including, but not limited to, modulation of apoptosis. These signaling pathways often comprise, but are by no means limited to, mechanisms which involve the activation or deactivation via phosphorylation state of an inhibitor peptide of NFkB (IkB), thus indirectly activating or deactivating NFkB.

As used herein, the term "JNK signaling pathway" refers to any one of the signaling pathways known in the art which involve the Jun amino terminal kinase (JNK) (Karin, 1998, *Cancer J from Scientific American*, 4:92-99; Wallach et al, 1999, *Ann Rev of Immunology*, 17:331-367). This kinase is generally responsive to a number of extracellular signals *e.g.* mitogens, cytokines, stress, and the like. The JNK signaling pathways mediate a range of cellular processes, including, but not limited to, modulation of apoptosis. In a preferred embodiment, JNK activation occurs through the activity of one or more members of the TRAF protein family (See, *e.g.*, Wajant et al, 1999, *Cytokine Growth Factor Rev* 10:15-26).

As used herein, the term "TGFβ signaling pathway" refers to any one of the signaling pathways known in the art which involve transforming growth factor beta. A TGFβ signaling pathway is initiated when this molecule binds to and induces a heterodimeric cell-surface complex consisting of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. This heterodimeric receptor then propagates the signal through phosphorylation of downstream target SMAD proteins. There are three functional classes of SMAD protein, receptor-regulated SMADs (R-SMADs), *e.g.*, SMAD2 and SMAD3, Co-mediator SMADs (Co-SMADs) and inhibitory SMADs (I-SMADs). Following phosphorylation by the heterodimeric receptor complex, the R-SMADs complex with the Co-SMAD and translocate to the nucleus, where in conjunction with other nuclear proteins, they regulate the transcription of target genes (Derynck, R., *et al.* (1998) *Cell* 95: 737-740). Reviewed in Massague, J. and Wotton, D. (2000) *EMBO J.* 19:1745.

The nucleotide sequence and amino acid sequnce of human SMAD2, is described in, for example, GenBank Accession No. gi:20127489. The nucleotide sequence and amino acid sequnce of murine SMAD2, is described in, for example, GenBank Accession No. gi:31560567. The nucleotide sequence and amino acid

sequnce of human SMAD3, is described in, for example, GenBank Accession No. gi:42476202. The nucleotide sequence and amino acid sequnce of murine SMAD3, is described in, for example, GenBank Accession No. gi:31543221.

"GATA3" is a Th2-specific transcription factor that is required for the development of Th2 cells. GATA-binding proteins constitute a family of transcription factors that recognize a target site conforming to the consensus WGATAR (W = A or T and R = A or G). GATA3 interacts with SMAD3 following its phosphorylation by TGFβ signaling to induce the differentiation of T helper cells. The nucleotide sequence and amino acid sequnce of human GATA3, is described in, for example, GenBank Accession Nos. gi:4503928 and gi:14249369. The nucleotide sequence and amino acid sequnce of murine GATA3, is described in, for example, GenBank Accession No. gi:40254638. The domains of GATA3 responsible for specific DNA-binding site recognition (amino acids 303 to 348) and trans activation (amino acids 30 to 74) have been identified. The signaling sequence for nuclear localization of human GATA-3 is a property conferred by sequences within and surrounding the amino finger (amino acids 249 to 311) of the protein. Exemplary genes whose transcription is regulated by GATA3 include IL-5, IL-12, IL-13, and IL-12Rβ2.

TGFß also plays a key role in osteoblast differentiation and bone development and remodeling. Osteoblasts secrete and deposit TGFß into the bone matrix and can respond to it, thus enabling possible autocrine modes of action. TGFß regulates the proliferation and differentiation of osteoblasts both in vitro and in vivo; however, the effects of TGFß on osteoblast differentiation depend on the extracellular milieu and the differentiation stage of the cells. TGFß stimulates proliferation and early osteoblast differentiation, while inhibiting terminal differentiation. Accordingly, TGFß has been reported to inhibit expression of alkaline phosphatase and osteocalcin, among other markers of osteoblast differentiation and function (Centrella et al., 1994 Endocr. Rev., 15, 27–39). Osteoblasts express cell surface receptors for TGFß and its known effectors, Smad2 and Smad3.

As used herein, "osteocalcin", also called bone Gla protein, is a vitamin K–dependent, calcium-binding bone protein, the most abundant noncollagen protein in bone. Osteocalcin is specifically expressed in differentiated osteoblasts and odontoblasts. The TGF-ß-mediated decrease of osteocalcin has been shown to occur at

the mRNA level and does not require new protein synthesis. It has also been shown that transcription from the osteocalcin promoter requires binding of the transcription factor CBFA1, also known as Runx2, to a response element, named OSE2, in the osteocalcin promoter.

Runx factors are DNA binding proteins that can facilitate tissue-specific gene activation or repression (Lutterbach, B., and S. W. Hiebert. (2000) Gene 245:223-235). Mammalian Runt-related genes are essential for blood, skeletal, and gastric development and are commonly mutated in acute leukemias and gastric cancers (Lund, A. H., and M. van Lohuizen. (2002) Cancer Cell. 1:213-215). Runx factors exhibit a tissue-restricted pattern of expression and are required for definitive hematopoiesis and osteoblast maturation. Runx proteins have recently been shown to interact through their C-terminal segment with Smads, a family of signaling proteins that regulate a diverse array of developmental and biological processes in response to transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) family of growth factors. Moreover, subnuclear distribution of Runx proteins is mediated by the nuclear matrix-targeting signal, a protein motif present in the C terminus of Runx factors. Importantly, in vivo osteogenesis requires the C terminus of Runx2 containing the overlapping subnuclear targeting signal and the Smad interacting domain. The Runx and Smad proteins are jointly involved in the regulation of phenotypic gene expression and lineage commitment. Gene ablation studies have revealed that both Runx proteins and Smads are developmentally involved in hematopoiesis and osteogenesis. Furthermore, Runx2 and the BMP-responsive Smads can induce osteogènesis in mesenchymal pluripotent cells.

"Runx2" is one of three mammalian homologues of the Drosophila transcription factors Runt and Lozenge (Daga, A., et al.(1996) Genes Dev. 10:1194-1205). Runx2 is also expressed in T lymphocytes and cooperates with oncogenes c-myc, p53, and Pim1 to accelerate T-cell lymphoma development in mice (Blyth, K., et al. (2001) Oncogene 20:295-302).

Runx2 expression also plays a key role in osteoblast differentiation and skeletal formation. In addition to osteocalcin, Runx2 regulates expression of several other genes that are activated during osteoblast differentiation, including alkaline

phosphatase, collagen, osteopontin, and osteoprotegerin ligand . These genes also contain Runx2 -binding sites in their promoters. These observations suggest that Runx2 is an essential transcription factor for osteoblast differentiation. This hypothesis is strongly supported by the absence of bone formation in mouse embryos in which the cbfa1 gene was inactivated. Furthermore, cleidocranial dysplasia, a human disorder in which some bones are not fully developed, has been associated with mutations in a cbfa1 allele. In addition to its role in osteoblast differentiation, Runx2 has been implicated in the regulation of bone matrix deposition by differentiated osteoblasts. The expression of Runx2 is regulated by factors that influence osteoblast differentiation. Accordingly, BMPs can activate, while Smad2 and glucocorticoids can inhibit, Runx2 expression. In addition, Runx2 can bind to an OSE2 element in its own promoter, suggesting the existence of an autoregulatory feedback mechanism of transcriptional regulation during osteoblast differentiation. For a review, see, Alliston, et al.(2000) *EMBO J* 20:2254.

The nucleotide sequence and amino acid sequnce of human Runx2, is described in, for example, GenBank Accession No. gi:10863884. The nucleotide sequence and amino acid sequnce of murine Runx2, is described in, for example, GenBank Accession No. gi:20806529.

As used herein, "AP-1" refers to the transcription factor activator protein 1 (AP-1) which is a family of DNA-binding factors that are composed of dimers of two proteins that bind to one another via a leucine zipper motif. The best characterized AP-1 factor comprises the proteins Fos and Jun. (Angel, P. and Karin, M. (1991) *Biochim. Biophys. Acta* 1072:129-157; Orengo, I. F. , Black, H. S. , *et al.* (1989) *Photochem. Photobiol.* 49:71-77; Curran, T. and Franza, B. R., Jr. (1988) *Cell* 55, 395-397). The AP-1 dimers bind to and transactivate promoter regions on DNA that contain cis-acting phorbol 12-tetradecanoate 13-acetate (TPA) response elements to induce transcription of genes involved in cell proliferation, metastasis, and cellular metabolism (Angel, P. , et al. (1987) Cell 49, 729-739. AP-1 is induced by a variety of stimuli and is implicated in the development of cancer and autoimmune disease. The nucleotide sequence and amino acid sequnce of human AP-1, is described in, for example, GenBank Accession No. gi:20127489.

As used herein, the term "nucleic acid" is intended to include fragments or equivalents thereof (e.g., fragments or equivalents thereof KRC, TRAF, c-Jun, c-Fos,

GATA3, Runx2, SMAD, GLα). The term "equivalent" is intended to include nucleotide sequences encoding functionally equivalent KRC proteins, i.e., proteins which have the ability to bind to the natural binding partner(s) of the KRC antigen. In a preferred embodiment, a functionally equivalent KRC protein has the ability to bind TRAF, *e.g.*, TRAF2, in the cytoplasm of an immune cell, *e.g.*, a T cell. In another preferred embodiment, a functionally equivalent KRC protein has the ability to bind Jun, *e.g.*, c-Jun, in the nucleoplasm of an immune cell, *e.g.*, a T cell. In another preferred embodiment, a functionally equivalent KRC protein has the ability to bind GATA3 in the nucleoplasm of an immune cell, *e.g.*, a T cell. In yet another preferred embodiment, a functionally equivalent KRC protein has the ability to bind SMAD, *e.g.*, SMAD2 and/or SMAD3, in the cytoplasm of an immune cell, *e.g.*, a B cell. In yet another preferred embodiment, a functionally equivalent KRC has the ability to bind Runx2 in the nucleoplasm of an immune cell, *e.g.*, a B cell.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid molecule) in the genomic DNA of the organism from which the nucleic acid molecule is derived.

As used herein, an "isolated protein" or "isolated polypeptide" refers to a protein or polypeptide that is substantially free of other proteins, polypeptides, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the KRC protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of KRC protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced.

The nucleic acids of the invention can be prepared, e.g., by standard recombinant DNA techniques. A nucleic acid of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which has been automated in commercially available DNA synthesizers (See *e.g.,* Itakura *et al.* U.S. Patent No. 4,598,049; Caruthers *et al.* U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.,* bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.,* non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.,* replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid molecule of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the

parent cell, but are still included within the scope of the term as used herein. Preferably a host cell is a mammalian cell, *e.g.*, a human cell. In particularly preferred embodiments, it is a epithelial cell.

As used herein, the term "transgenic cell" refers to a cell containing a transgene.

As used herein, a "transgenic animal" includes an animal, *e.g.*, a non-human mammal, *e.g.*, a swine, a monkey, a goat, or a rodent, *e.g.*, a mouse, in which one or more, and preferably essentially all, of the cells of the animal include a transgene. The transgene is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, *e.g.*, by microinjection, transfection or infection, *e.g.*, by infection with a recombinant virus. The term genetic manipulation includes the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA.

As used herein, the term "rodent" refers to all members of the phylogenetic order *Rodentia*.

As used herein, the term "misexpression" includes a non-wild type pattern of gene expression. Expression as used herein includes transcriptional, post transcriptional, *e.g.*, mRNA stability, translational, and post translational stages. Misexpression includes: expression at non-wild type levels, *i.e.*, over or under expression; a pattern of expression that differs from wild type in terms of the time or stage at which the gene is expressed, *e.g.*, increased or decreased expression (as compared with wild type) at a predetermined developmental period or stage; a pattern of expression that differs from wild type in terms of decreased expression (as compared with wild type) in a predetermined cell type or tissue type; a pattern of expression that differs from wild type in terms of the splicing size, amino acid sequence, post-translational modification, or biological activity of the expressed polypeptide; a pattern of expression that differs from wild type in terms of the effect of an environmental stimulus or extracellular stimulus on expression of the gene, *e.g.*, a pattern of increased or decreased expression (as compared with wild type) in the presence of an increase or decrease in the strength of the stimulus. Misexpression includes any expression from a transgenic nucleic acid. Misexpression includes the lack or non-expression of a gene or

transgene, *e.g.*, that can be induced by a deletion of all or part of the gene or its control sequences.

As used herein, the term "knockout" refers to an animal or cell therefrom, in which the insertion of a transgene, *e.g.*, an exogenous DNA molecule, disrupts an endogenous gene in the animal or cell therefrom. This disruption can essentially eliminate KRC in the animal or cell. In preferred embodiments, misexpression of the gene encoding the KRC protein is caused by disruption of the KRC gene. For example, the KRC gene can be disrupted through removal of DNA encoding all or part of the protein.

In preferred embodiments, the animal can be heterozygous or homozygous for a misexpressed KRC gene, *e.g.*, it can be a transgenic animal heterozygous or homozygous for a KRC transgene.

In preferred embodiments, the animal is a transgenic mouse with a transgenic disruption of the KRC gene, preferably an insertion or deletion, which inactivates the gene product.

In another aspect, the invention features, a nucleic acid molecule which, when introduced into an animal or cell, results in the misexpression of the KRC gene in the animal or cell. In preferred embodiments, the nucleic acid molecule, includes a KRC nucleotide sequence which includes a disruption, *e.g.*, an insertion or deletion and preferably the insertion of a marker sequence. The nucleotide sequence of the wild type KRC is known in the art and described in, for example, Mak, C.H., *et al.* (1998) *Immunogenetics* 48:32-39, the contents of which are incorporated herein by reference.

As used herein, the term "marker sequence" refers to a nucleic acid molecule that (a) is used as part of a nucleic acid construct (*e.g.*, the targeting construct) to disrupt the expression of the gene of interest (*e.g.*, the KRC gene) and (b) is used to identify those cells that have incorporated the targeting construct into their genome. For example, the marker sequence can be a sequence encoding a protein which confers a detectable trait on the cell, such as an antibiotic resistance gene, *e.g.*, neomycin resistance gene, or an assayable enzyme not typically found in the cell, *e.g.*, alkaline phosphatase, horseradish peroxidase, luciferase, beta-galactosidase and the like.

As used herein, "disruption of a gene" refers to a change in the gene sequence, *e.g.*, a change in the coding region. Disruption includes: insertions, deletions,

point mutations, and rearrangements, *e.g.*, inversions. The disruption can occur in a region of the native KRC DNA sequence (*e.g.*, one or more exons) and/or the promoter region of the gene so as to decrease or prevent expression of the gene in a cell as compared to the wild-type or naturally occurring sequence of the gene. The "disruption" can be induced by classical random mutation or by site directed methods. Disruptions can be transgenically introduced. The deletion of an entire gene is a disruption. Preferred disruptions reduce KRC levels to about 50% of wild type, in heterozygotes or essentially eliminate KRC in homozygotes.

As used herein, the term "antibody" is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which binds (immunoreacts with) an antigen, such as Fab and $F(ab')_2$ fragments, single chain antibodies, intracellular antibodies, scFv, Fd, or other fragments. Preferably, antibodies of the invention bind specifically or substantially specifically to KRC, TRAF, c-Jun, c-Fos, GATA3, SMAD, or Runx2, molecules (*i.e.*, have little to no cross reactivity with non-KRC, non-TRAF, non-c-Jun, non-c-Fos, non-GATA3, non-SMAD, or non-Runx2, molecules). The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody compositions thus typically display a single binding affinity for a particular antigen with which it immunoreacts.

As used herein, the term "disorders that would benefit from the modulation of KRC activity or expression" or "KRC associated disorder" includes disorders in which KRC activity is aberrant or which would benefit from modulation of a KRC activity. Preferably, KRC associated disorders involve aberrant proliferation of cells, *e.g.*, excessive or unwanted proliferation of cells or deficient proliferation of cells. In one embodiment, KRC associated disorders are disorders such as inflammation. Examples of KRC associated disorders include: disorders involving aberrant or unwanted proliferation of cells, *e.g.*, inflammation, autoimmunity, neoplasia, or cell

death, e.g., apoptosis, or necrosis. KRC associated disorders may also include disorders that have been linked generally to aberrant TGFβ activity or function, including, for example, B cell chronic lymphocytic leukemia (B-CLL). Further examples of KRC associated disorders include carcinomas, adenocarcinomas, leukemias, lymphomas, and other neoplasias. KRC disorders may also include disorders that have been linked generally to aberrant TNF receptor activity or function, including Crohn's Disease (Baert and Rutgeerts, 1999, *Int J Colorectal Dis*, 14:47-51) and certain cardiovascular diseases (Ferrari, 1999, *Pharmacol Res*, 40:97-105). They may also include disorders characterized by uncontrolled or aberrant levels of apoptosis, for example myelokathexis (Aprikyan *et al.*, 2000, *Blood*, 95:320-327), and autoimmune lymphoproliferative syndrome (Jackson and Puck, 1999, *Curr Op Pediatr*, 11:521-527; Straus *et al.*, 1999, *Ann Intern Med*, 130:591-601). KRC associated disorders may also include metabolic bone disorders, such as, but not limited to, osteoporosis, osteomalacia, skeletal changes of hyperparathyroidism and chronic renal failure (renal osteodystrophy) and osteitis deformans (Paget's disease of bone).

In one embodiment, small molecules can be used as test compounds. The term "small molecule" is a term of the art and includes molecules that are less than about 7500, less than about 5000, less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (e.g., Cane et al. 1998. Science 282:63), and natural product extract libraries. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic. For example, a small molecule is preferably not itself the product of transcription or translation.

Various aspects of the invention are described in further detail below:

II. Screening Assays to Identify KRC Modulating Agents

Modulators of KRC activity can be known (*e.g.*, dominant negative inhibitors of KRC activity, antisense KRC intracellular antibodies that interfere with

KRC activity, peptide inhibitors derived from KRC) or can be identified using the methods described herein. The invention provides methods (also referred to herein as "screening assays") for identifying other modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptidomimetics, small molecules or other drugs) which modulate KRC activity and for testing or optimizing the activity of other agents.

For example, in one embodiment, molecules which bind, e.g., to KRC or a molecule in a signaling pathway involving KRC (e.g., TRAF, NF-kB, JNK, GATA3, SMAD, Runx2, or AP-1)or have a stimulatory or inhibitory effect on the expression and or activity of KRC or a molecule in a signal transduction pathway involving KRC can be identified. For example, c-Jun, NF-kB, TRAF, GATA3, SMAD, Runx2, and JNK function in a signal transduction pathway involving KRC, therefore, any of these molecules can be used in the subject screening assays. Although the specific embodiments described below in this section and in other sections may list one of these molecules as an example, other molecules in a signal transduction pathway involving KRC can also be used in the subject screening assays.

In one embodiment, the ability of a compound to directly modulate the expression, post-translational modification (*e.g.*, phosphorylation), or activity of KRC is measured in an indicator composition using a screening assay of the invention.

The indicator composition can be a cell that expresses the KRC protein or a molecule in a signal transduction pathway involving KRC, for example, a cell that naturally expresses or, more preferably, a cell that has been engineered to express the protein by introducing into the cell an expression vector encoding the protein. Preferably, the cell is a mammalian cell, e.g., a human cell. In one embodiment, the cell is a T cell. In another embodiment, the cell is a B cell. In another embodiment, the cell is a osteoblast. Alternatively, the indicator composition can be a cell-free composition that includes the protein (*e.g.*, a cell extract or a composition that includes e.g., either purified natural or recombinant protein).

Compounds identified using the assays described herein can be useful for treating disorders associated with aberrant expression, post-translational modification, or activity of KRC or a molecule in a signaling pathway involving KRC *e.g*: disorders that would benefit from modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway, modulation of an NFkB signaling pathway,

modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx2, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription (Igα chain germline transcription), and/or modulation of osteocalcin gene transcription.

Conditions that can benefit from modulation of a signal transduction pathway involving KRC include autoimmune disorders as well as malignancies, immunodeficiency disorders and metabolic bone diseases.. Compounds which modulate KRC expression and/or activity can also be used to modulate the immune response.

The subject screening assays can be performed in the presence or absence of other agents. In one embodiment, the subject assays are performed in the presence of an agent that provides a T cell receptor-mediated signal. In another embodiment, the subject assays are performed in the presence of an agent that provides a B cell receptor-mediated signal

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell-free assay, and the ability of the agent to modulate the activity of KRC or a molecule in a signal transduction pathway involving KRC can be confirmed *in vivo*, *e.g.*, in an animal such as an animal model for multiple myeloma, neoplastic diseases, renal cell carcinoma, B-CLL, metabolic bone disease, or autoimmune diseases.

Moreover, a modulator of KRC or a molecule in a signaling pathway involving KRC identified as described herein (*e.g.*, an antisense nucleic acid molecule, or a specific antibody, or a small molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such a modulator. Alternatively, a modulator identified as described herein can be used in an animal model to determine the mechanism of action of such a modulator.

In another embodiment, it will be understood that similar screening assays can be used to identify compounds that indirectly modulate the activity and/or

expression of KRC *e.g.*, by performing screening assays such as those described above using molecules with which KRC interacts, *e.g.*, molecules that act either upstream or downstream of KRC in a signal transduction pathway.

The cell based and cell free assays of the invention are described in more detail below.

A. Cell Based Assays

The indicator compositions of the invention can be cells that expresses at least one of a KRC protein or non-KRC protein in the KRC signaling pathway (such as, e.g., TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1) for example, a cell that naturally expresses the endogenous molecule or, more preferably, a cell that has been engineered to express an exogenous KRC, TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 protein by introducing into the cell an expression vector encoding the protein(s). Alternatively, the indicator composition can be a cell-free composition that includes at least one of a KRC or a non- KRC protein such as TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 (*e.g.*, a cell extract from a cell expressing the protein or a composition that includes purified KRC, TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 protein, either natural or recombinant protein).

Compounds that modulate expression and/or activity of KRC, or a non-KRC protein that acts upstream or downstream of can be identified using various "read-outs."

For example, an indicator cell can be transfected with an expression vector, incubated in the presence and in the absence of a test compound, and the effect of the compound on the expression of the molecule or on a biological response regulated by can be determined. The biological activities of include activities determined *in vivo*, or *in vitro*, according to standard techniques. Activity can be a direct activity, such as an association with a target molecule or binding partner (*e.g.*, a protein such as the Jun, *e.g.*, c-Jun, TRAF, *e.g.*, TRAF2, GATA3, SMAD, *e.g.*, SMAD2, SMAD3, protein. Alternatively, activity is an indirect activity, such as a cellular signaling activity occurring downstream of the interaction of the protein with an target molecule or a biological effect occurring as a result of the signaling cascade triggered by that

interaction. For example, biological activities of KRC described herein include: modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway, modulation of an NFkB signaling pathway, modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF2, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD3, modulation of degradation of Runx 2, modulation of degradation of GATA3, modulation of effector T cell function, modulation of T cell anergy, modulation of apoptosis, or modulation of T cell differentiation, and/or modulation of IgA germline transcription.

To determine whether a test compound modulates KRC protein expression, *in vitro* transcriptional assays can be performed. In one example of such an assay, a regulatory sequence (eg., the full length promoter and enhancer) of KRC can be operably linked to a reporter gene such as chloramphenicol acetyltransferase (CAT), GFP, or luciferase and introduced into host cells. Other techniques are known in the art.

As used interchangeably herein, the terms "operably linked" and "operatively linked" are intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence in a host cell (or by a cell extract). Regulatory sequences are art-recognized and can be selected to direct expression of the desired protein in an appropriate host cell. The term regulatory sequence is intended to include promoters, enhancers, polyadenylation signals and other expression control elements. Such regulatory sequences are known to those skilled in the art and are described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transfected and/or the type and/or amount of protein desired to be expressed.

A variety of reporter genes are known in the art and are suitable for use in the screening assays of the invention. Examples of suitable reporter genes include those which encode chloramphenicol acetyltransferase, beta-galactosidase, alkaline

phosphatase, green fluorescent protein, or luciferase. Standard methods for measuring the activity of these gene products are known in the art.

A variety of cell types are suitable for use as an indicator cell in the screening assay. Preferably a cell line is used which expresses low levels of endogenous KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2) and is then engineered to express recombinant protein. Cells for use in the subject assays include both eukaryotic and prokaryotic cells. For example, in one embodiment, a cell is a bacterial cell. In another embodiment, a cell is a fungal cell, such as a yeast cell. In another embodiment, a cell is a vertebrate cell, *e.g.*, an avian cell or a mammalian cell (*e.g.*, a murine cell, or a human cell).

In one embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is higher than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that stimulates the expression of KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2). In another embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is lower than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that inhibits the expression of KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2).

In one embodiment, the invention provides methods for identifying compounds that modulate cellular responses in which KRC is involved.

In one embodiment differentiation of cells, e.g., T cells, can be used as an indicator of modulation of KRC or a signal transduction pathway involving KRC. Cell differentiation can be monitored directly (*e.g.* by microscopic examination of the cells for monitoring cell differentiation), or indirectly, *e.g.*, by monitoring one or more markers of cell differentiation (*e.g.*, an increase in mRNA for a gene product associated with cell differentiation, or the secretion of a gene product associated with cell differentiation, such as the secretion of a protein (*e.g.*, the secretion of cytokines) or the expression of a cell surface marker (such as CD69). Standard methods for detecting mRNA of interest, such as reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting, are known in the art. Standard methods for detecting protein

secretion in culture supernatants, such as enzyme linked immunosorbent assays (ELISA), are also known in the art. Proteins can also be detected using antibodies, *e.g.*, in an immunoprecipitation reaction or for staining and FACS analysis.

In another embodiment, the ability of a compound to modulate effector T cell function can be determined. For example, in one embodiment, the ability of a compound to modulate T cell proliferation, cytokine production, and/or cytotoxicity can be measured using techniques well known in the art.

In one embodiment, the ability of a compound to modulate IL-2 production can be determined. Production of IL-2 can be monitored, for example, using Northern or Western blotting. IL-2 can also be detected using an ELISA assay or in a bioassay, *e.g.*, employing cells which are responsive to IL-2 (*e.g.*, cells which proliferate in response to the cytokine or which survive in the presence of the cytokine) using standard techniques.

In another embodiment, the ability of a compound to modulate apoptosis can be determined. Apoptosis can be measured in the presence or the absence of Fas-mediated signals. In one embodiment, cytochrome C release from mitochondria during cell apoptosis can be detected, *e.g.*, plasma cell apoptosis (as described in, for example, Bossy-Wetzel E. *et al.* (2000) *Methods in Enzymol.* 322:235-42). Other exemplary assays include: cytofluorometric quantitation of nuclear apoptosis induced in a cell-free system (as described in, for example, Lorenzo H.K. *et al.* (2000) *Methods in Enzymol.* 322:198-201); apoptotic nuclease assays (as described in, for example, Hughes F.M. (2000) *Methods in Enzymol.* 322:47-62); analysis of apoptotic cells, *e.g.*, apoptotic plasma cells, by flow and laser scanning cytometry (as described in, for example, Darzynkiewicz Z. *et al.* (2000) *Methods in Enzymol.* 322:18-39); detection of apoptosis by annexin V labeling (as described in, for example, Bossy-Wetzel E. *et al.* (2000) *Methods in Enzymol.* 322:15-18); transient transfection assays for cell death genes (as described in, for example, Miura M. *et al.* (2000) *Methods in Enzymol.* 322:480-92); and assays that detect DNA cleavage in apoptotic cells, *e.g.*, apoptotic plasma cells (as described in, for example, Kauffman S.H. *et al.* (2000) *Methods in Enzymol.* 322:3-15). Apoptosis can also be measured by propidium iodide staining or by TUNEL assay. In

another embodiment, the transcription of genes associated with a cell signaling pathway involved in apoptosis (*e.g.*, JNK) can be detected using standard methods.

In another embodiment, mitochondrial inner membrane permeabilization can be measured in intact cells by loading the cytosol or the mitochondrial matrix with a die that does not normally cross the inner membrane, e.g., calcein (Bernardi et al. 1999. Eur. J. Biochem. 264:687; Lemasters, J., J. et al. 1998. Biochem. Biophys. Acta 1366:177. In another embodiment, mitochondrial inner membrane permeabilization can be assessed, e.g., by determining a change in the mitochondrial inner membrane potential ($\Delta\Psi m$). For example, cells can be incubated with lipophilic cationic fluorochromes such as DiOC6 (Gross et al. 1999. Genes Dev. 13:1988) (3,3'dihexyloxacarbocyanine iodide) or JC-1 (5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide). These dyes accumulate in the mitochondrial matrix, driven by the Ψm . Dissipation results in a reduction of the fluorescence intensity (e.g., for DiOC6 (Gross et al. 1999. Genes Dev. 13:1988) or a shift in the emission spectrum of the dye. These changes can be measured by cytofluorometry or microscopy.

In yet another embodiment, the ability of a compound to modulate translocation of KRC to the nucleus can be determined. Translocation of KRC to the nucleus can be measured, e.g., by nuclear translocation assays in which the emission of two or more fluorescently-labeled species is detected simultaneously. For example, the cell nucleus can be labeled with a known fluorophore specific for DNA, such as Hoechst 33342. The KRC protein can be labeled by a variety of methods, including expression as a fusion with GFP or contacting the sample with a fluorescently-labeled antibody specific for KRC. The amount KRC that translocates to the nucleus can be determined by determining the amount of a first fluorescently-labeled species, i.e., the nucleus, that is distributed in a correlated or anti-correlated manner with respect to a second fluorescently-labeled species, i.e., KRC, as described in U.S. Patent No. 6,400,487, the contents of which are hereby incorporated by reference.

In one embodiment, the effect of a compound on a JNK signaling pathway can be determined. The JNK group of MAP kinases is activated by exposure of cells to environmental stress or by treatment of cells with pro-inflammatory cytokines. A combination of studies involving gene knockouts and the use of dominant-negative

mutants have implicated both MKK4 and MKK7 in the phosphorylation and activation of JNK. Targets of the JNK signal transduction pathway include the transcription factors ATF2 and c-Jun. JNK binds to an NH_2-terminal region of ATF2 and c-Jun and phosphorylates two sites within the activation domain of each transcription factor, leading to increased transcriptional activity. JNK is activated by dual phosphorylation on Thr-183 and Tyr-185. To determine the effect of a compound on a JNK signal transduction pathway, the ability of the compound to modulate the activation status of various molecules in the signal transduction pathway can be determined using standard techniques. For example, in one embodiment, the phosphorylation status of JNK can be examined by immunoblotting with the anti-ACTIVE-JNK antibody (Promega), which specifically recognizes the dual phosphorylated TPY motif.

In another embodiment, the effect of a compound on an NFkB signal transduction pathway can be determined. The ability of the compound to modulate the activation status of various components of the NFkB pathway can be determined using standard techniques. NFkB constitutes a family of Rel domain-containing transcription factors that play essential roles in the regulation of inflammatory, anti-apoptotic, and immune responses. The function of the NFkB/Rel family members is regulated by a class of cytoplasmic inhibitory proteins termed IBs that mask the nuclear localization domain of NFkB causing its retention in the cytoplasm. Activation of NFkB by TNF-α and IL-1 involves a series of signaling intermediates, which may converge on the NFkB-inducing kinase (NIK). This kinase in turn activates the IB kinase (IKK) isoforms. These IKKs phosphorylate the two regulatory serines located in the N termini of IB molecules, triggering rapid ubiquitination and degradation of IB in the 26S proteasome complex. The degradation of IB unmasks a nuclear localization signal present in the NFkB complex, allowing its rapid translocation into the nucleus, where it engages cognate B enhancer elements and modulates the transcription of various NFkB-responsive target genes. In one embodiment, the ability of a compound to modulate one or more of: the status of NFkB inhibitors, the ability of NFkB to translocate to the nucleus, or the activation of NFkB dependent gene transcription can be measured.

In one embodiment, the ability of a compound to modulate AP-1 activity can be measured. The AP-1 complex is comprised of the transcription factors Fos and Jun. The AP-1 complex activity is controlled by regulation of Jun and Fos transcription

and by posttranslation modification, for example, the activation of several MAPKS, ERK, p38 and JN, is required for AP-1 transcriptional activity. In one embodiment, the modulation of transcription mediated by AP-1 can be measured. In another embodiment, the ability of a compound to modulate the activity of AP-1, e.g., by modulating its phosphorylation or its ubiquitination can be measured. In one embodiment, the ubiquitination of AP-1 can be measured using techniques known in the art. In another embodiment, the degradation of AP-1 (or of c-Jun and/or c-Fos) can be measured using known techniques.

The loss of AP-1 has been associated with T cell anergy. Accordingly, in one embodiment, the ability of a test compound to modulate T cell anergy can be determined, e.g, by assaying secondary T cell responses. If the T cells are unresponsive to the secondary activation attempts, as determined by IL-2 synthesis and/or T cell proliferation, a state of anergy or has been induced. Standard assay procedures can be used to measure T cell anergy, for example, T cell proliferation can be measured, for example, by assaying [^{3}H] thymidine incorporation. In another embodiment, signal transduction can be measured, e.g., activation of members of the MAP kinase cascade or activation of the AP-1 complex can be measured. In another embodiment, intracellular calcium mobilization, protein levels members of the NFAT cascade can be measured.

In another embodiment, the effect of a compound on Ras and Rac activity can be measured using standard techniques. In one embodiment, actin polymerization, e.g., by measuring the immunofluorescence of F-actin can be measured.

In another embodiment, the effect of the compound on ubiquitination of, for example, AP1, SMAD, TRAF, and/or Runx2, can be measured, by, for example in vitro or in vivo ubiquitination assays. In vitro ubiquitination assays are described in, for example, Fuchs, S. Y., Bet al. (1997) J. Biol. Chem. 272:32163-32168. In vivo ubiquitination assays are described in, for example, Treier, M., L. et al. (1994) Cell 78:787-798.

In another embodiment, the effect of the compound on the degradation of, for example, a KRC target molecule and/or a KRC binding partner, can be measured by, for example, coimmunoprecipitation of KRC, *e.g.*, full-length KRC and/or a fragment thereof, with, *e.g.*, SMAD, GATA3, Runx2, TRAF, Jun, and/or Fos. Western blotting of the coimmunoprecipitate and probing of the blots with antibodies to KRC and the KRC

target molecule and/or a KRC binding partner can be quantitated to determine whether degradation has occurred.

In one embodiment, the ability of the compound to modulate TGFβ signaling in B cells can be measured. For example, as described herein, KRC is a coactivator of GLα promoter activity and a corepressor of the osteocalcin gene. In the absence of KRC, GLα transcription is diminished in B cells, and osteocalcin gene transcription is augmented in osteoblasts. Accordingly, in one embodiment, the ability of the compound to modulate TGFβ signaling in B cells can be measured by measuring the transcription of GLα. In another embodiment, osteocalcin gene transcription can be measured. In one embodiment, RT-PCR is used to measure the transcritpion. Furthermore, given the ability of KRC to interact with SMAD and drive the transcription of a SMAD reporter construct, the ability of a compound to modulate TGFβ signaling in B cells can be measured by measuring the transcriptional ability of SMAD. In one embodiment, SMAD, or a fragment thereof, *e.g.*, a basic SMAD-binding element, is operably linked to a luciferase reporter gene. Other TGFβ regulated genes are known in the art (e.g., Massague and Wotton. 2000 EMBO 19:1745.)

The ability of the test compound to modulate KRC (or a molecule in a signal transduction pathway involving to KRC) binding to a substrate or target molecule (e.g., TRAF, GATA3, SMAD, Runx2, or Jun in the case of KRC) can also be determined. Determining the ability of the test compound to modulate KRC binding to a target molecule (*e.g.*, a binding partner such as a substrate) can be accomplished, for example, by coupling the target molecule with a radioisotope or enzymatic label such that binding of the target molecule to KRC or a molecule in a signal transduction pathway involving KRC can be determined by detecting the labeled KRC target molecule in a complex. Alternatively, KRC be coupled with a radioisotope or enzymatic label to monitor the ability of a test compound to modulate KRC binding to a target molecule in a complex. Determining the ability of the test compound to bind to KRC can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to KRC can be determined by detecting the labeled compound in a complex. For example, targets can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively,

compounds can be labeled, e.g., with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

In another embodiment, the ability of KRC or a molecule in a signal transduction pathway involving KRC to be acted on by an enzyme or to act on a substrate can be measured. For example, in one embodiment, the effect of a compound on the phosphorylation of KRC can be measured using techniques that are known in the art.

It is also within the scope of this invention to determine the ability of a compound to interact with KRC or a molecule in a signal transduction pathway involving KRC without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with a KRC molecule without the labeling of either the compound or the molecule (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and

Exemplary target molecules of KRC include: Jun, TRAF (e.g., TRAF2) GATA3, SMAD, *e.g.,* SMAD2 and SMAD3, and Runx2.

In another embodiment, a different (*i.e.*, non-KRC) molecule acting in a pathway involving KRC that acts upstream or downstream of KRC can be included in an indicator composition for use in a screening assay. Compounds identified in a screening assay employing such a molecule would also be useful in modulating KRC activity, albeit indirectly. For example, the ability of TRAF (e.g., TRAF2) to activate NFKβ dependent gene expression can be measured, or the ability of SMAD to activate TGFβ-dependent gene transcription can be measured..

The cells used in the instant assays can be eukaryotic or prokaryotic in origin. For example, in one embodiment, the cell is a bacterial cell. In another embodiment, the cell is a fungal cell, *e.g.*, a yeast cell. In another embodiment, the cell is a vertebrate cell, *e.g.*, an avian or a mammalian cell. In a preferred embodiment, the cell is a human cell.

The cells of the invention can express endogenous KRC or another protein in a signaling pathway involving KRC or can be engineered to do so. For example, a cell that has been engineered to express the KRC protein and/or a non protein which acts upstream or downstream of can be produced by introducing into the cell an expression vector encoding the protein.

Recombinant expression vectors that can be used for expression of KRC or a molecule in a signal transduction pathway involving KRC (e.g., a protein which acts upstream or downstream of KRC) are known in the art. For example, the cDNA is first introduced into a recombinant expression vector using standard molecular biology techniques. A cDNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR) or by screening an appropriate cDNA library. The nucleotide sequences of cDNAs for or a molecule in a signal transduction pathway involving (*e.g.*, human, murine and yeast) are known in the art and can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods.

Following isolation or amplification of a cDNA molecule encoding KRC or a non-KRC molecule in a signal transduction pathway involving KRC the DNA fragment is introduced into an expression vector. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may

be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid molecule in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression and the level of expression desired, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell, those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences) or those which direct expression of the nucleotide sequence only under certain conditions (*e.g.*, inducible regulatory sequences).

When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma virus, adenovirus, cytomegalovirus and Simian Virus 40. Non-limiting examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). A variety of mammalian expression vectors carrying different regulatory sequences are commercially available. For constitutive expression of the nucleic acid in a mammalian host cell, a preferred regulatory element is the cytomegalovirus promoter/enhancer. Moreover, inducible regulatory systems for use in mammalian cells are known in the art, for example systems in which gene expression is regulated by heavy metal ions (see *e.g.*, Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42; Searle *et al.*

(1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, e.d. Nouer, L. , CRC, Boca Raton , FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel & Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Still further, many tissue-specific regulatory sequences are known in the art, including the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916) and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Vector DNA can be introduced into mammalian cells via conventional transfection techniques. As used herein, the various forms of the term "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into mammalian host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these

integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on a separate vector from that encoding KRC or, more preferably, on the same vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

In one embodiment, within the expression vector coding sequences are operatively linked to regulatory sequences that allow for constitutive expression of the molecule in the indicator cell (*e.g.*, viral regulatory sequences, such as a cytomegalovirus promoter/enhancer, can be used). Use of a recombinant expression vector that allows for constitutive expression of KRC or a molecule in a signal transduction pathway involving KRC in the indicator cell is preferred for identification of compounds that enhance or inhibit the activity of the molecule. In an alternative embodiment, within the expression vector the coding sequences are operatively linked to regulatory sequences of the endogenous gene for KRC or a molecule in a signal transduction pathway involving KRC (*i.e.*, the promoter regulatory region derived from the endogenous gene). Use of a recombinant expression vector in which expression is controlled by the endogenous regulatory sequences is preferred for identification of compounds that enhance or inhibit the transcriptional expression of the molecule.

In yet another aspect of the invention, the KRC protein or fragments thereof can be used as "bait protein" e.g., in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with KRC ("binding proteins" or " bp") and are involved in KRC activity. Such KRC -binding proteins are also likely to be involved in the propagation of signals by the KRC proteins or KRC targets such as, for example, downstream elements of an KRC-mediated signaling pathway. Alternatively, such KRC -binding proteins can be KRC inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for an KRC protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming an KRC dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the KRC protein or a molecule in a signal transduction pathway involving KRC.

B. Cell-free assays

In another embodiment, the indicator composition is a cell free composition. KRC or a non- KRC protein in a signal transduction pathway involving KRC expressed by recombinant methods in a host cells or culture medium can be isolated from the host cells, or cell culture medium using standard methods for protein purification. For example, ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies can be used to produce a purified or semi-purified protein that can be used in a cell free composition. Alternatively, a lysate or an extract of cells expressing the protein of interest can be prepared for use as cell-free composition.

In one embodiment, compounds that specifically modulate KRC activity or the activity of a molecule in a signal transduction pathway involving KRC are identified based on their ability to modulate the interaction of KRC with a target molecule to which KRC binds. The target molecule can be a DNA molecule, *e.g.*, an KRC -responsive element, such as the regulatory region of a chaperone gene) or a protein molecule. Suitable assays are known in the art that allow for the detection of

protein-protein interactions (*e.g.*, immunoprecipitations, two-hybrid assays and the like) or that allow for the detection of interactions between a DNA binding protein with a target DNA sequence (*e.g.*, electrophoretic mobility shift assays, DNAse I footprinting assays and the like). By performing such assays in the presence and absence of test compounds, these assays can be used to identify compounds that modulate (*e.g.*, inhibit or enhance) the interaction of KRC with a target molecule.

In one embodiment, the amount of binding of KRC or a molecule in a signal transduction pathway involving KRC to the target molecule in the presence of the test compound is greater than the amount of binding of KRC to the target molecule in the absence of the test compound, in which case the test compound is identified as a compound that enhances binding of KRC to a target. In another embodiment, the amount of binding of the KRC to the target molecule in the presence of the test compound is less than the amount of binding of the KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2) to the target molecule in the absence of the test compound, in which case the test compound is identified as a compound that inhibits binding of KRC to the target. Binding of the test compound to KRC or a molecule in a signal transduction pathway involving KRC can be determined either directly or indirectly as described above. Determining the ability of KRC protein to bind to a test compound can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In the methods of the invention for identifying test compounds that modulate an interaction between KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2) protein and a target molecule, the complete KRC protein can be used in the method, or, alternatively, only portions of the protein can be used. For example, an isolated KRC interacting domain (e.g., consisting of amino acids 204-1055 or a larger subregion including an interacting domain) can be used. An assay can be used to identify test compounds that either stimulate or inhibit the interaction between the KRC protein and a target molecule. A test compound that stimulates the interaction between the protein and

a target molecule is identified based upon its ability to increase the degree of interaction between, e.g., KRC and a target molecule as compared to the degree of interaction in the absence of the test compound and such a compound would be expected to increase the activity of KRC in the cell. A test compound that inhibits the interaction between the protein and a target molecule is identified based upon its ability to decrease the degree of interaction between the protein and a target molecule as compared to the degree of interaction in the absence of the compound and such a compound would be expected to decrease KRC activity.

In one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either KRC (or a molecule in a signal transduction pathway involving KRC, e.g., Jun, TRAF, GATA3, SMAD, Runx2) or a respective target molecule for example, to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, or to accommodate automation of the assay. Binding of a test compound to a KRC or a molecule in a signal transduction pathway involving KRC, or interaction of an KRC protein (or a molecule in a signal transduction pathway involving KRC) with a target molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided in which a domain that allows one or both of the proteins to be bound to a matrix is added to one or more of the molecules. For example, glutathione-S-transferase fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or KRC protein, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix is immobilized in the case of beads, and complex formation is determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either an KRC protein or a molecule in a signal transduction pathway involving KRC, or a target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which are reactive with protein or target molecules but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and unbound target or KRC protein is trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with KRC or a molecule in a signal transduction pathway involving KRC or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the KRC protein or target molecule.

C. Assays Using Knock-Out Cells

In another embodiment, the invention provides methods for identifying compounds that modulate a biological effect of KRC or a molecule in a signal transduction pathway involving KRC using cells deficient in KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2). As described in the Examples, inhibition of KRC activity (*e.g.*, by disruption of the KRC gene) in cells results, e.g., in a deficiency of IL-2 production, impaired Th2 cell development, and/or impaired TGFβR signaling. Thus, cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be used identify agents that modulate a biological response regulated by KRC by means other than modulating KRC itself (*i.e.*, compounds that "rescue" the KRC deficient phenotype). Alternatively, a "conditional knock-out" system, in which the gene is rendered non-functional in a conditional manner, can be used to create deficient cells for use in screening assays. For example, a tetracycline-regulated system for conditional disruption of a gene as described in WO 94/29442 and U.S. Patent No. 5,650,298 can be used to create cells, or animals from which cells can be isolated, be rendered deficient in KRC(or a molecule in a signal transduction pathway involving KRC e.g., Jun, TRAF,

GATA3, SMAD, Runx2) in a controlled manner through modulation of the tetracycline concentration in contact with the cells. Specific cell types, e.g., lymphoid cells (*e.g.*, thymic, splenic and/or lymph node cells) or purified cells such as T cells, B cells, osteoblasts, osteoclasts, from such animals can be used in screening assays. In one embodiment, the entire 5.4 kB exon 2 of KRC can be replaced, e.g., with a neomycin cassette, resulting in an allele that produces no KRC protein. This embodiment is described in the appended examples.

In the screening method, cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be contacted with a test compound and a biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC can be monitored. Modulation of the response in cells deficient in KRC or a molecule in a signal transduction pathway involving KRC (as compared to an appropriate control such as, for example, untreated cells or cells treated with a control agent) identifies a test compound as a modulator of the KRC regulated response.

In one embodiment, the test compound is administered directly to a non-human knock out animal, preferably a mouse (*e.g.*, a mouse in which the KRC gene or a gene in a signal transduction pathway involving KRC is conditionally disrupted by means described above, or a chimeric mouse in which the lymphoid organs are deficient in KRC or a molecule in a signal transduction pathway involving KRC as described above), to identify a test compound that modulates the *in vivo* responses of cells deficient in KRC. In another embodiment, cells deficient in KRC are isolated from the non-human KRC deficient animal or a molecule in a signal transduction pathway involving KRC deficient animal, and contacted with the test compound *ex vivo* to identify a test compound that modulates a response regulated by KRC in the cells

Cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be obtained from a non-human animals created to be deficient in KRC or a molecule in a signal transduction pathway involving KRC Preferred non-human animals include monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep. In preferred embodiments, the deficient animal is a mouse. Mice deficient in KRC or a molecule in a signal transduction pathway involving KRC can be made using methods known in the art. One example of such a method and the resulting KRC heterozygous and homozygous animals is described in the appended examples. Non-human animals

deficient in a particular gene product typically are created by homologous recombination. In an exemplary embodiment, a vector is prepared which contains at least a portion of the gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the endogenous KRC. The gene preferably is a mouse gene. For example, a mouse KRC gene can be isolated from a mouse genomic DNA library using the mouse KRC cDNA as a probe. The mouse KRC gene then can be used to construct a homologous recombination vector suitable for modulating an endogenous KRC gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector).

Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous KRC protein). In the homologous recombination vector, the altered portion of the gene is flanked at its 5' and 3' ends by additional nucleic acid of the gene to allow for homologous recombination to occur between the exogenous gene carried by the vector and an endogenous gene in an embryonic stem cell. The additional flanking nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced gene has homologously recombined with the endogenous gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing

homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In one embodiment of the screening assay, compounds tested for their ability to modulate a biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC are contacted with deficient cells by administering the test compound to a non-human deficient animal *in vivo* and evaluating the effect of the test compound on the response in the animal.

The test compound can be administered to a non-knock out animal as a pharmaceutical composition. Such compositions typically comprise the test compound and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal compounds, isotonic and absorption delaying compounds, and the like, compatible with pharmaceutical administration. The use of such media and compounds for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or compound is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. Pharmaceutical compositions are described in more detail below.

In another embodiment, compounds that modulate a biological response regulated by KRC or a signal transduction pathway involving KRC are identified by contacting cells deficient in KRC *ex vivo* with one or more test compounds, and determining the effect of the test compound on a read-out. In one embodiment, KRC deficient cells contacted with a test compound *ex vivo* can be readministered to a subject.

For practicing the screening method *ex vivo*, cells deficient, e.g., in KRC, Jun, TRAF, GATA3, SMAD, and/or Runx, can be isolated from a non-human deficient animal or embryo by standard methods and incubated (*i.e.*, cultured) *in vitro* with a test compound. Cells (*e.g.*, T cells, B cells, and/or osteoblasts) can be isolated from e.g., KRC, Jun, TRAF, GATA3, SMAD, and/or Runx, deficient animals by standard techniques. In another embodiment, the cells are isolated form animals deficient in one or more of KRC, Jun, TRAF, GATA3, SMAD, and/or Runx.

In another embodiment, cells deficient in more than one member of a signal transduction pathway involving KRC can be used in the subject assays.

Following contact of the deficient cells with a test compound (either *ex vivo* or *in vivo*), the effect of the test compound on the biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC can be determined by any one of a variety of suitable methods, such as those set forth herein, *e.g.*, including light microscopic analysis of the cells, histochemical analysis of the cells, production of proteins, induction of certain genes, e.g., cytokine gene, such as IL-2, degradation of certain proteins, *e.g.*, ubiquitination of certain proteins, as described herein.

D. Test Compounds

A variety of test compounds can be evaluated using the screening assays described herein. The term "test compound" includes any reagent or test agent which is employed in the assays of the invention and assayed for its ability to influence the expression and/or activity of KRC or a molecule in a signal transduction pathway involving KRC. More than one compound, *e.g.*, a plurality of compounds, can be tested at the same time for their ability to modulate the expression and/or activity of, e.g., KRC in a screening assay. The term "screening assay" preferably refers to assays which test the ability of a plurality of compounds to influence the readout of choice rather than to tests which test the ability of one compound to influence a readout. Preferably, the subject assays identify compounds not previously known to have the effect that is being screened for. In one embodiment, high throughput screening can be used to assay for the activity of a compound.

In certain embodiments, the compounds to be tested can be derived from libraries (*i.e.*, are members of a library of compounds). While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures of other compounds, such as benzodiazepines (Bunin *et al.* (1992). *J. Am. Chem. Soc.* 114:10987; DeWitt *et al.* (1993). *Proc. Natl. Acad. Sci. USA* 90:6909) peptoids (Zuckermann. (1994). *J. Med. Chem.* 37:2678) oligocarbamates (Cho *et al.* (1993). *Science.* 261:1303-), and hydantoins (DeWitt *et al. supra*). An

approach for the synthesis of molecular libraries of small organic molecules with a diversity of 104-105 as been described (Carell *et al.* (1994). *Angew. Chem. Int. Ed. Engl.* 33:2059- ; Carell *et al.* (1994) Angew. *Chem. Int. Ed. Engl.* 33:2061-).

The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145). Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: Erb *et al.* (1994). *Proc. Natl. Acad. Sci. USA* 91:11422- ; Horwell *et al.* (1996) *Immunopharmacology* 33:68- ; and in Gallop *et al.* (1994); *J. Med. Chem.* 37:1233-.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); In still another embodiment, the combinatorial polypeptides are produced from a cDNA library.

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

Candidate/test compounds include, for example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, *e.g.*, Lam, K.S. *et al.* (1991) *Nature* 354:82-84; Houghten, R. *et al.* (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids; 2) phosphopeptides (*e.g.*, members of random and partially degenerate, directed phosphopeptide libraries, see, *e.g.*, Songyang, Z. *et al.* (1993) *Cell* 72:767-778); 3) antibodies (*e.g.*, polyclonal, monoclonal, humanized, anti-

idiotypic, chimeric, and single chain antibodies as well as Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments of antibodies); 4) small organic and inorganic molecules (*e.g.*, molecules obtained from combinatorial and natural product libraries); 5) enzymes (*e.g.*, endoribonucleases, hydrolases, nucleases, proteases, synthatases, isomerases, polymerases, kinases, phosphatases, oxido-reductases and ATPases), and 6) mutant forms of KRC (*e.g.*, dominant negative mutant forms of the molecule).

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

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Compounds identified in the subject screening assays can be used in methods of modulating one or more of the biological responses regulated by KRC. It will be understood that it may be desirable to formulate such compound(s) as pharmaceutical compositions (described supra) prior to contacting them with cells.

Once a test compound is identified that directly or indirectly modulates, e.g., KRC expression or activity, or a molecule in a signal transduction pathway involving KRC, by one of the variety of methods described hereinbefore, the selected test compound (or "compound of interest") can then be further evaluated for its effect on cells, for example by contacting the compound of interest with cells either *in vivo* (*e.g.*, by administering the compound of interest to a subject) or *ex vivo* (*e.g.*, by isolating cells from the subject and contacting the isolated cells with the compound of interest or, alternatively, by contacting the compound of interest with a cell line) and determining the effect of the compound of interest on the cells, as compared to an appropriate control (such as untreated cells or cells treated with a control compound, or carrier, that does not modulate the biological response).

The instant invention also pertains to compounds identified in the subject screening assays.

III. Pharmaceutical Compositions

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and compounds for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water,

Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition will preferably be sterile and should be fluid to the extent that easy syringability exists. It will preferably be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal compounds, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic compounds, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an compound which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding compounds, and/or adjuvant materials can be included as part of the

composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating compound such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening compound such as sucrose or saccharin; or a flavoring compound such as peppermint, methyl salicylate, or orange flavoring.

In one embodiment, the test compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from, e.g., Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

IV. Methods for Modulating Biological Responses Regulated by KRC

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant KRC expression and/or activity. For example, an immune system disorder or condition associated with an undesirable immune response (such as an unwanted or excessive inflammatory response, an autoimmune disorder, graft-versus-host disease (GVHD), an allogeneic transplant) or an immune system disorder or condition that would benefit from an enhanced immune response, e.g. an immunosuppressed individual.

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant or unwanted immune response by administering an agent that downmodulates the expression and/or activity of KRC or,

alternatively, an abnormally low immune response, by administering to the subject an agent which upmodulates the activity of KRC. Subjects at risk for such disorders can be identified by, for example, any or a combination of diagnostic or prognostic assays known in the art. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the aberrant immune response, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of immune response aberrancy, for example, a KRC antagonist or agonist agent can be used for treating a subject. The appropriate agent can be determined based on screening assays described herein. In a preferred embodiment, the agent may be a peptide comprising the amino acid residues 204-1055 of KRC, a peptide that binds to KRC, a KRC ZAS domain or a small molecule.

Another aspect of the invention pertains to methods of modulating KRC activity for therapeutic purposes. KRC activity can be modulated in order to modulate the immune response. Because KRC upregulates immune responses, enhanced KRC activity and/or expression results in upregulation of immune responses, whereas inhibition of KRC activity results in downregulation of immune responses.

Modulatory methods of the invention involve contacting a cell (*e.g.*, a T cell B cell, and/or osteoblast) with a agent that modulates the activity of KRC. An agent that modulates KRC activity can be an agent as described herein, such as a KRC peptide (*e.g.*, the agent may be a peptide comprising the amino acid residues 204-1055 of KRC, a peptide that binds to KRC, a KRC ZAS domain or a small molecule), a nucleic acid molecule encoding one of the aforementioned peptides, a KRC agonist or antagonist, a peptidomimetic of a KRC agonist or antagonist, a KRC peptidomimetic, or other small molecule identified using the screening methods described herein.

These modulatory methods can be performed *in vitro* (*e.g.*, by contacting the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a condition or disorder that would benefit from up- or down-modulation of a KRC polypeptide, *e.g.*, a disorder characterized by an unwanted, insufficient, or aberrant immune response. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) KRC activity.

Inhibition of KRC activity is desirable in situations in which KRC is abnormally upregulated and/or in which decreased KRC activity is likely to have a beneficial effect, for example in a situation of an excessive or unwanted immune response. Such situations include conditions, disorders, or diseases such as an autoimmune disorder, a transplant (*e.g.*, a bone marrow transplant, a stem cell transplant, a heart transplant, a lung transplant, a liver transplant, a kidney transplant, a cornea transplant, or a skin transplant), graft versus host disease (GVHD), an allergy, a metabolic bone disease, or an inflammatory disorder. Likewise, upregulation of KRC activity is desirable in situations in which KRC is abnormally downregulated and/or in which increased KRC activity is likely to have a beneficial effect (*e.g.*, in a neoplasia).

As used herein, the term "autoimmunity" refers to the condition in which a subject's immune system starts reacting against his or her own tissues. Non-limiting examples of autoimmune diseases and disorders having an autoimmune component that would benefit from modulation of a KRC activity include type 1 diabetes, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

The terms "neoplasia," "hyperplasia," and "tumor" are often commonly referred to as "cancer," which is a general name for more than 100 disease that are characterized by uncontrolled, abnormal growth of cells. Examples of malignancies include but are not limited to acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related lymphoma; B cell chronic lymphocytic

leukemia; cancer of the bile duct; bladder cancer; bone cancer, osteosarcomal malignant fibrous histiocytomal brain stem gliomal brain tumor; breast cancer; bronchial adenomas; carcinoid tumors; adrenocortical carcinoma; central nervous system lymphoma; cancer of the sinus, cancer of the gall bladder; gastric cancer; cancer of the salivary glands; cancer of the esophagus; neural cell cancer; intestinal cancer (e.g., of the large or small intestine); cervical cancer; colon cancer; colorectal cancer; cutaneous T-cell lymphoma; B-cell lymphoma; T-cell lymphoma; endometrial cancer; epithelial cancer; endometrial cancer; intraocular melanoma; retinoblastoma; hairy cell leukemia; liver cancer; Hodgkin's disease; Kaposi's sarcoma; acute lymphoblastic leukemia; lung cancer; non-Hodgkin's lymphoma; melanoma; multiple myeloma; neuroblastoma; prostate cancer; retinoblastoma; Ewing's sarcoma; vaginal cancer; Waldenstrom's macroglobulinemia; adenocarcinomas; ovarian cancer, chronic lymphocytic leukemia, pancreatic cancer; and Wilm's tumor.

Exemplary agents for use in upmodulating KRC (*i.e.*, KRC agonists) include, *e.g.*, nucleic acid molecules encoding KRC polypeptides, KRC peptides, and compounds that stimulate the interaction of KRC with TRAF, GATA3, SMAD, Runx, GLα, c-Jun, for example (*e.g.*, compounds identified in the subject screening assays).

Exemplary agents for use in downmodulating KRC (*i.e.*, KRC antagonists) include agents that inhibit the activity of KRC in cell, (*e.g.*, compounds identified in the subject screening assays).

A. Downregulation of KRC Biological Activities

There are numerous embodiments of the invention for downregulating the function of a KRC polypeptide to thereby downregulate immune responses. Downregulation can be in the form of inhibiting or blocking an immune response already in progress, or may involve preventing the induction of an immune response. The functions of activated immune cells can be inhibited by downregulating immune cell responses or by inducing specific anergy in immune cells, or both.

For example, KRC activity can be inhibited by contacting a cell which expresses KRC with an agent that inhibits KRC. Such an agent can be a compound identified by the screening assays described herein. In another embodiment, the agent is

a peptide. In a preferred embodiment, the agent can interact with the amino acid residues 204-1055 of KRC to inhibit KRC activity.

An immune response can be further inhibited by the use of an additional agent that can thereby downmodulate the immune response, as described further herein.

Agents that inhibit a KRC activity can be identified by their ability to inhibit immune cell proliferation and/or effector function, or to induce anergy when added to an *in vitro* assay. A number of art-recognized readouts of cell activation can be employed to measure, *e.g.*, cell proliferation or effector function (*e.g.*, cytokine production or phagocytosis) in the presence of the activating agent. The ability of a test agent to block this activation can be readily determined by measuring the ability of the agent to effect a decrease in proliferation or effector function being measured.

In another embodiment, immune responses can be downregulated in a subject by removing immune cells from the patient, contacting the immune cells *in vitro* with an agent (*e.g.*, a small molecule) that downregulates KRC activity, and reintroducing the *in vitro*-stimulated immune cells into the patient.

Downregulating immune responses by inhibiting KRC activity is useful in downmodulating the immune response, *e.g.*, in situations of tissue, skin and organ transplantation, in graft-versus-host disease (GVHD), or allergies, or in autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis. For example, blockage of immune cell function results in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by immune cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits the activity of KRC, *e.g.*, by blocking the interaction of KRC with, for example, TRAF, Jun, GATA3, SMAD, or Runx2, in immune cells (such as a KRC, TRAF, Jun, GATA3, SMAD, or Runx2 peptide or a small molecule) alone or in conjunction with another downmodulatory agent can inhibit the generation of an immune response. Moreover, inhibition of KRC activity by inhibition of, for example, KRC-TRAF interaction may also be sufficient to anergize the immune cells, thereby inducing tolerance in a subject.

Other downmodulatory agents that can be used in connection with the downmodulatory methods of the invention include, for example, blocking antibodies

against other immune cell markers, or soluble forms of other receptor ligand pairs (*e.g.*, agents that disrupt the interaction between CD40 and CD40 ligand (*e.g.*, anti CD40 ligand antibodies)), antibodies against cytokines, or immunosuppressive drugs (*e.g.*, FK506, cyclosporin, rapamycin, steroids).

For example, inhibition of KRC activity may also be useful in treating autoimmune disease. Many autoimmune disorders are the result of inappropriate activation of immune cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive immune cells may reduce or eliminate disease symptoms. Administration of agents that inhibit an activity of KRC may lead to long-term relief from the disease. Additionally, co-administration of agents which block costimulation of immune cells by disrupting receptor-ligand interactions may be useful in inhibiting immune cell activation to prevent production of autoantibodies or cytokines which may be involved in the disease process. The efficacy of reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in MRL/*lpr/lpr* mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

Inhibition of immune cell activation is useful therapeutically in the treatment of allergies and allergic reactions, *e.g.*, by inhibiting IgE production. An agent that inhibits KRC activity can be administered to an allergic subject to inhibit immune cell-mediated allergic responses in the subject. Inhibition of KRC activity can be accompanied by exposure to allergen in conjunction with appropriate MHC molecules. Allergic reactions can be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, immune cell-mediated allergic responses can be inhibited locally or systemically by administration of an agent that inhibits KRC activity.

Downregulation of immune cell activation through inhibition of KRC activity may also be important therapeutically in pathogenic infections of immune cells (*e.g.*, by viruses or bacteria). For example, in the acquired immune deficiency syndrome

(AIDS), viral replication is stimulated by immune cell activation. Inhibition of KRC activity may result in inhibition of viral replication and thereby ameliorate the course of AIDS.

Downregulation of immune cell activation via inhibition of KRC activity interaction may also be useful in treating inflammatory disorders and in promoting the maintenance of pregnancy when there exists a risk of immune-mediated spontaneous abortion. Inhibition of KRC activity may also be useful for the treatment of disorders in which bone mass, bone mineral density and bone formation are impaired, *e.g.*, a metabolic bone disease, *e.g.*, osteoporosis.

Exemplary Inhibitory Compounds

Since inhibition of KRC activity is associated with an decreased immune response, to downmodulate or inhibit the immune response, cells (*e.g.*, T cells) are contacted with an agent that inhibits KRC activity. The immune cells may be contacted with the agent *in vitro* and then the cells can be administered to a subject or, alternatively, the agent may be administered to the subject (*e.g.*, directly to an articular site at which T growth and/or differentiation is desired). The methods of the invention using KRC inhibitory compounds can be used in the treatment of disorders in which the immune response is diminished, blocked, inhibited, downregulated or the like. Inhibitory compounds of the invention can be, for example, intracellular binding molecules that act to specifically inhibit the expression or activity of KRC. As used herein, the term "intracellular binding molecule" is intended to include molecules that act intracellularly to inhibit the expression or activity of a protein by binding to the protein or to a nucleic acid (*e.g.*, an mRNA molecule) that encodes the protein. Examples of intracellular binding molecules, described in further detail below, include antisense nucleic acids, intracellular antibodies, peptidic compounds that inhibit the interaction of KRC with a target molecule (*e.g.*, calcineurin) and chemical agents that specifically inhibit KRC activity.

i. Antisense Nucleic Acid Molecules

In one embodiment, an inhibitory compound of the invention is an antisense nucleic acid molecule that is complementary to a gene encoding KRC, or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a particular protein in a cell is well known in the art (see *e.g.*, Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986; Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule comprises a nucleotide sequence that is complementary to the coding strand of another nucleic acid molecule (*e.g.*, an mRNA sequence) and accordingly is capable of hydrogen bonding to the coding strand of the other nucleic acid molecule. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the coding region and an untranslated region (*e.g.*, at the junction of the 5' untranslated region and the coding region). Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated region of an mRNA.

Given the coding strand sequences encoding KRC disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of KRC mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of KRC mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of KRC mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and

enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a KRC protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid

molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).
In another embodiment, an antisense nucleic acid of the invention is a compound that mediates RNAi. RNA interfering agents include, but are not limited to, nucleic acid molecules including RNA molecules which are homologous to the target gene or genomic sequence, *e.g.*, KRC, c-Jun, c-Fos, GATA3, SMAD, and/or Runx2, or a fragment thereof, "short interfering RNA" (siRNA), "short hairpin" or "small hairpin RNA" (shRNA), and small molecules which interfere with or inhibit expression of a target gene by RNA inerference (RNAi). RNA interference is a post-transcriptional, targeted gene-silencing technique that uses double-stranded RNA (dsRNA) to degrade messenger RNA (mRNA) containing the same sequence as the dsRNA (Sharp, P.A. and Zamore, P.D. 287, 2431-2432 (2000); Zamore, P.D., *et al. Cell* 101, 25-33 (2000). Tuschl, T. *et al. Genes Dev.* 13, 3191-3197 (1999)). The process occurs when an endogenous ribonuclease cleaves the longer dsRNA into shorter, 21- or 22-nucleotide-long RNAs, termed small interfering RNAs or siRNAs. The smaller RNA segments then mediate the degradation of the target mRNA. Kits for synthesis of RNAi are commercially available from, *e.g.* New England Biolabs and Ambion. In one

embodiment one or more of the chemistries described above for use in antisense RNA can be employed.

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach, 1988, *Nature* 334:585-591) can be used to catalytically cleave KRC mRNA transcripts to thereby inhibit translation of KRC mRNA. A ribozyme having specificity for a KRC-encoding nucleic acid can be designed based upon the nucleotide sequence of SEQ ID NO:1 a nucleic acid molecule encoding another KRC family polypeptide. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a KRC-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, KRC mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W., 1993, *Science* 261:1411-1418.

Alternatively, gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of KRC (*e.g.*, the KRC promoter and/or enhancers) to form triple helical structures that prevent transcription of the KRC gene in target cells. See generally, Helene, C., 1991, *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.*, 1992, *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J., 1992, *Bioassays* 14(12):807-15.

In yet another embodiment, the KRC nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup B. *et al.*, 1996, *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for

specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. *et al.*, 1996, *supra*; Perry-O'Keefe *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93: 14670-675.

PNAs of KRC nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of KRC nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup B., 1996, *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup B. *et al.*, 1996, *supra*; Perry-O'Keefe *supra*).

In another embodiment, PNAs of KRC can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of KRC nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNAse H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B., 1996, *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B., 1996, *supra* and Finn P.J. *et al.*, 1996, *Nucleic Acids Res.* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.*, 1989, *Nucleic Acid Res.* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P.J. *et al.*, 1996, *supra*). Alternatively, chimeric molecules can be

synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.*, 1975, *Bioorganic Med. Chem. Lett.* 5: 1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, *Proc. Natl. Acad. Sci. US.* 86:6553-6556; Lemaitre *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.*, 1988, *Bio-Techniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell. Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in the culture medium *in vitro* or in the circulatory system or in interstitial fluid *in vivo*. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species.

ii. Intracellular Antibodies

Another type of inhibitory compound that can be used to inhibit the expression and/or activity of KRC protein in a cell is an intracellular antibody specific for KRC discussed herein. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.*

269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

To inhibit protein activity using an intracellular antibody, a recombinant expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell. For inhibition of transcription factor activity according to the inhibitory methods of the invention, preferably an intracellular antibody that specifically binds the transcription factor is expressed within the nucleus of the cell. Nuclear expression of an intracellular antibody can be accomplished by removing from the antibody light and heavy chain genes those nucleotide sequences that encode the N-terminal hydrophobic leader sequences and adding nucleotide sequences encoding a nuclear localization signal at either the N- or C-terminus of the light and heavy chain genes (see *e.g.*, Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551). A preferred nuclear localization signal to be used for nuclear targeting of the intracellular antibody chains is the nuclear localization signal of SV40 Large T antigen (see Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Mhashilkar, A. M. *et al.* (1995) *EMBO J.* 14:1542-1551).

To prepare an intracellular antibody expression vector, antibody light and heavy chain cDNAs encoding antibody chains specific for the target protein of interest, *e.g.*, KRC protein, is isolated, typically from a hybridoma that secretes a monoclonal antibody specific for KRC protein. Preparation of antisera against KRC protein has been described in the art (see *e.g.*, Rao *et al*, U.S. patent 5,656,452). Anti-KRC protein antibodies can be prepared by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with a KRC protein immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed KRC protein or a chemically synthesized KRC peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory compound. Antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497) (see also,

Brown *et al.* (1981) *J. Immunol* 127:539-46; Brown *et al.* (1980) *J Biol Chem* 255:4980-83; Yeh *et al.* (1976) *PNAS* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75). The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.*, 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a KRC protein immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds specifically to the KRC protein. Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-KRC protein monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:550-52; Gefter *et al. Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinary skilled artisan will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody that specifically binds the maf protein are identified by screening the hybridoma culture supernatants for such antibodies, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody that binds to a KRC can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with the protein, or a peptide thereof, to thereby isolate immunoglobulin library members that bind specifically to the protein. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP*™ *Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and compounds particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; Barbas *et al.* (1991) *PNAS* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Once a monoclonal antibody of interest specific for KRC has been identified (*e.g.*, either a hybridoma-derived monoclonal antibody or a recombinant antibody from a combinatorial library, including monoclonal antibodies to KRC that are already known in the art), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from the display package (*e.g.*, phage) isolated during the library screening process. Nucleotide sequences of antibody light and heavy chain genes from which PCR primers or cDNA library probes can be prepared are known in the art. For example, many such

sequences are disclosed in Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database.

Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods. As discussed above, the sequences encoding the hydrophobic leaders of the light and heavy chains are removed and sequences encoding a nuclear localization signal (*e.g.*, from SV40 Large T antigen) are linked in-frame to sequences encoding either the amino- or carboxy terminus of both the light and heavy chains. The expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes full-length antibody light and heavy chains such that a full-length antibody is expressed intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed intracellularly. In the most preferred embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible peptide linker (*e.g.*, $(Gly_4Ser)_3$) and expressed as a single chain molecule. To inhibit transcription factor activity in a cell, the expression vector encoding the KRC-specific intracellular antibody is introduced into the cell by standard transfection methods as described hereinbefore.

iii. *KRC-Derived Peptidic Compounds*

In another embodiment, an inhibitory compound of the invention is a peptidic compound derived from the KRC amino acid sequence. In particular, the inhibitory compound comprises a portion of KRC (or a mimetic thereof) that mediates interaction of KRC with a target molecule such that contact of KRC with this peptidic compound competitively inhibits the interaction of KRC with the target molecule. In an exemplary embodiment, the peptide compound is designed based on the region of KRC that mediates interaction of KRC with, for example, TRAF, Jun, GATA3, SMAD, and/or Runx2. As described herein, amino acid residues 204-1055 of the KRC protein mediate the interaction of the KRC proteins with TRAF and peptides spanning the region inhibit the ability of TRAF to bind to and phosphorylate KRC proteins, without affecting the phosphatase activity of TRAF against other substrates. Moreover, when

expressed intracellularly, peptides spanning this region inhibit KRC dephosphorylation, nuclear translocation and KRC-mediated gene expression in response to stimulation, thereby inhibiting KRC-dependent functions.

In a preferred embodiment, a KRC inhibitory compound is a peptidic compound, which is prepared based on a TRAF-interacting region of KRC. A peptide can be derived from the TRAF-interacting region of KRC having an amino acid sequence that comprises the amino acid residues 204-1055 of KRC. In another preferred embodiment, a KRC inhibitory compound is a peptidic compound, which is prepared based on a c-Jun-interacting region of KRC. A peptide can be derived from the c-Jun-interacting region of KRC having an amino acid sequence that comprises the amino acid residues 204-1055 of KRC. Alternatively, longer or shorter regions of human KRC can be used such as a peptide.

The peptidic compounds of the invention can be made intracellularly in immune cells by introducing into the immune cells an expression vector encoding the peptide. Such expression vectors can be made by standard techniques, using, for example, oligonucleotides that encode the amino acid sequences of SEQ ID NO: 2. The peptide can be expressed in intracellularly as a fusion with another protein or peptide (*e.g.*, a GST fusion). Alternative to recombinant synthesis of the peptides in the cells, the peptides can be made by chemical synthesis using standard peptide synthesis techniques. Synthesized peptides can then be introduced into cells by a variety of means known in the art for introducing peptides into cells (*e.g.*, liposome and the like).

Other inhibitory agents that can be used to specifically inhibit the activity of an KRC protein are chemical compounds that directly inhibit KRC activity or inhibit the interaction between KRC and target molecules. Such compounds can be identified using screening assays that select for such compounds, as described in detail above.

B. Upregulation of KRC Biological Activities

Stimulation of KRC activity as a means of upregulating immune responses is also useful in therapy. Upregulation of immune responses can be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through enhancing of KRC activity is useful in cases of infections with microbes, *e.g.*, bacteria, viruses, or parasites. For

example, in one embodiment, an agent that enhances KRC activity, *e.g.*, a small molecule or a KRC peptide, is therapeutically useful in situations where upregulation of antibody and cell-mediated responses, resulting in more rapid or thorough clearance of a virus, would be beneficial. These conditions include viral skin diseases such as Herpes or shingles, in which case such an agent can be delivered topically to the skin. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of such agents systemically. In certain instances, it may be desirable to further administer other agents that upregulate immune responses, for example, agents that transduce signals via costimulatory receptors, in order further augment the immune response.

Alternatively, immune responses can be enhanced in an infected patient by removing immune cells from the patient, contacting immune cells *in vitro* with an agent (*e.g.*, a small molecule) that enhances KRC activity, and reintroducing the *in vitro*-stimulated immune cells into the patient. In another embodiment, a method of enhancing immune responses involves isolating infected cells from a patient, *e.g.*, virally infected cells, transfecting them with a nucleic acid molecule encoding a form of KRC that is more active than the wild type KRC, such that the cells express all or a portion of the KRC molecule on their surface, and reintroducing the transfected cells into the patient. The transfected cells may be capable of preventing an inhibitory signal to, and thereby activating, immune cells *in vivo*.

An agent that enhances KRC activity can be used prophylactically in therapy against various polypeptides, *e.g.*, polypeptides derived from pathogens for vaccination. Immunity against a pathogen, *e.g.*, a virus, can be induced by vaccinating with a viral polypeptide along with an agent that enhances KRC activity. Nucleic acid vaccines can be administered by a variety of means, for example, by injection (*e.g.*, intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes *et al.* (1996) *J. Biotechnol.* 44:37)). Alternatively, nucleic acid vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, *e.g.*, Peyers patches by oral delivery of DNA (Schubbert (1997)

Proc. Natl. Acad. Sci. USA 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces (Sizemore *et al.* (1995) *Science* 270:29).

Stimulation of an immune response to tumor cells can also be achieved by enhancing KRC activity by treating a patient with an agent that for example, enhancing KRC-TRAF interaction. Preferred examples of such agents include, *e.g.,* and compounds identified in the subject screening assays and peptides.

In another embodiment, the immune response can be stimulated by enhancing of KRC activity such that preexisting tolerance is overcome. For example, immune responses against antigens to which a subject cannot mount a significant immune response, *e.g.,* tumor-specific antigens, can be induced by administering an agent that stimulates the activity of KRC activity. Other KRC agonists can be used as adjuvants to boost responses to foreign antigens in the process of active immunization.

In another embodiment, KRC activity is increased in order to increase IgA production. IgA is the primary Ig isotype induced at the mucosal surface and the mucosal immune system represents the first line of immunological defense against pathogens encountering the mucosal surfaces of the body. Secretory IgA (S-IgA) in mucosal secretions provides protection against bacterial and viral pathogens and neutralizes microbial toxins. S-IgA binds protein Ags, thus limiting their absorption, and helps to prevent allergies and other hypersensitivity reactions. As demonstrated herein, animals deficient in KRC activity and/or expression have impaired IgA production. Accordingly, increasing KRC activity and/or expression would be beneficial to increase passive immunity against bacterial and viral pathogens.

In one embodiment, immune cells are obtained from a subject and cultured *ex vivo* in the presence of an agent that that enhances KRC activity to expand the population of immune cells. In a further embodiment the immune cells are then administered to a subject. immune cells can be stimulated to proliferate *in vitro* by, for example, providing the immune cells with a primary activation signal and a costimulatory signal, as is known in the art. Various forms of KRC polypeptides or agents that enhance KRC activity can also be used to costimulate proliferation of immune cells. In one embodiment immune cells are cultured *ex vivo* according to the

method described in PCT Application No. WO 94/29436. The agent can be soluble, attached to a cell membrane or attached to a solid surface, such as a bead.

In an additional embodiment, in performing any of the methods described herein, it is within the scope of the invention to upregulate an immune response by administering one or more additional agents. For example, the use of other agents known to stimulate the immune response, such as cytokines, adjuvants, or stimulatory forms of costimulatory molecules or their ligands can be used in conjunction with an agent that enhances KRC activity.

In another embodiment, a method of upregulating immune responses involves transfecting them with a nucleic acid molecule encoding a KRC molecule with a mutation or a peptide that enhances, for example, KRC-TRAF interaction (*e.g.*, a TRAF-C domain), such that the cells express the KRC molecule (*e.g.*, in the cell membrane) or the peptide (*e.g.*, in the cytoplasm), and reintroducing the transfected cells into the patient. The ability of the transfected cells to be activated can thus be increased.

Examples of other immunomodulating reagents include antibodies that provide a costimulatory signal, (*e.g.*, agonists of CD28 or ICOS), stimulating antibodies against immune cell markers , and/or cytokines and the like.

Exemplary Stimulatory Compounds

Since upregulation of KRC activity is associated with an increased immune response, a compound that specifically stimulates KRC activity and/or expression can be used to enhance or upmodulate an immune response. In the stimulatory methods of the invention, a subject is treated with a stimulatory compound that stimulates expression and/or activity of a KRC molecule. The methods of the invention using KRC stimulatory compounds can be used in the treatment of disorders in which the immune response is enhanced, promoted, stimulated, upregulated or the like.

Examples of stimulatory compounds include active KRC protein, expression vectors encoding KRC and chemical agents that specifically stimulate KRC activity.

A preferred stimulatory compound is a nucleic acid molecule encoding KRC, wherein the nucleic acid molecule is introduced into the subject (*e.g.*, T cells of

the subject) in a form suitable for expression of the KRC protein in the cells of the subject. For example, a KRC cDNA (full length or partial KRC cDNA sequence) is cloned into a recombinant expression vector and the vector is transfected into the immune cell using standard molecular biology techniques. The KRC cDNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR) or by screening an appropriate cDNA library. The nucleotide sequences of KRC cDNA is known in the art and can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods.

Following isolation or amplification of KRC cDNA, the DNA fragment is introduced into a suitable expression vector, as described above. Nucleic acid molecules encoding KRC in the form suitable for expression of the KRC in a host cell, can be prepared as described above using nucleotide sequences known in the art. The nucleotide sequences can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods.

Another form of a stimulatory compound for stimulating expression of KRC in a cell is a chemical compound that specifically stimulates the expression or activity of endogenous KRC in the cell. Such compounds can be identified using screening assays that select for compounds that stimulate the expression or activity of KRC as described herein.

The method of the invention for modulating KRC activity in a subject can be practiced either *in vitro* or *in vivo* (the latter is discussed further in the following subsection). For practicing the method *in vitro*, cells (*e.g.*, T cells) can be obtained from a subject by standard methods and incubated (*i.e.*, cultured) *in vitro* with a stimulatory or inhibitory compound of the invention to stimulate or inhibit, respectively, the activity of KRC. Methods for isolating immune cells are known in the art.

Cells treated *in vitro* with either a stimulatory or inhibitory compound can be administered to a subject to influence the growth and/or differentiation of immune cells in the subject. For example, immune cells can be isolated from a subject, expanded in number *in vitro* by enhancing KRC activity in the cells using an enhancing agent (thereby promoting the proliferation of the cells), and then the immune cells can be

readministered to the same subject, or another subject tissue compatible with the donor of the immune cells. Accordingly, in another embodiment, the modulatory method of the invention comprises culturing immune cells *in vitro* with a KRC modulator and further comprises administering the immune cells to a subject to thereby modulate T growth and/or differentiation in a subject. Upon culture *in vitro*, the immune cells can differentiate into mature immune cells and thus the methods encompass administering this mature immune cells to the subject. For administration of cells or T to a subject, it may be preferable to first remove residual compounds in the culture from the cells or T before administering them to the subject. This can be done for example by gradient centrifugation of the cells or by washing of the T tissue. For further discussion of *ex vivo* genetic modification of cells followed by readministration to a subject, see also U.S. Patent No. 5,399,346 by W.F. Anderson *et al.*

In other embodiments, a stimulatory or inhibitory compound is administered to a subject *in vivo*, such as directly to an articulation site of a subject. For stimulatory or inhibitory agents that comprise nucleic acids (*e.g.*, recombinant expression vectors encoding KRC, antisense RNA, intracellular antibodies or KRC-derived peptides), the compounds can be introduced into cells of a subject using methods known in the art for introducing nucleic acid (*e.g.*, DNA) into cells *in vivo*. Examples of such methods include:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly injecting the DNA into the cells (see *e.g.*, Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (*e.g.*, a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (*e.g.*, from BioRad).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by

intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991) *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 *etc.*) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al. Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985)

Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (*e.g.,* Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product, such as an enzymatic assay.

V. Diagnostic Assays

In another aspect, the invention features a method of diagnosing a subject for a disorder associated with aberrant biological activity or KRC (*e.g.*, that would benefit from modulation of, e.g., modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway, modulation of an NFkB signaling pathway, modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF2, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD3, modulation of degradation of Runx 2, modulation of degradation of GATA3, modulation of effector T cell function, modulation of T cell anergy, modulation of apoptosis, or modulation of T cell differentiation, and/or modulation of IgA germline transcription.

In one embodiment, the invention comprises identifying the subject as one that would benefit from modulation of an KRC activity, e.g., modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway,

modulation of an NFkB signaling pathway, modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF2, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD3, modulation of degradation of Runx 2, modulation of degradation of GATA3, modulation of effector T cell function, modulation of T cell anergy, modulation of apoptosis, or modulation of T cell differentiation, and/or modulation of IgA germline transcription. For example, in one embodiment, expression of KRC or a molecule in a signal transduction pathway involving KRC can be detected in cells of a subject suspected of having a disorder associated with aberrant biological activity of KRC. The expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of said subject could then be compared to a control and a difference in expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject as compared to the control could be used to diagnose the subject as one that would benefit from modulation of an KRC activity.

The "change in expression" or "difference in expression" of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject can be, for example, a change in the level of expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject as compared to a previous sample taken from the subject or as compared to a control, which can be detected by assaying levels of, e.g., KRC mRNA, for example, by isolating cells from the subject and determining the level of KRC mRNA expression in the cells by standard methods known in the art, including Northern blot analysis, microarray analysis, reverse-transcriptase PCR analysis and *in situ* hybridizations. For example, a biological specimen can be obtained from the patient and assayed for, e.g., expression or activity of KRC or a molecule in a signal transduction pathway involving KRC. For instance, a PCR assay could be used to measure the level of KRC in a cell of the subject. A level of KRC higher or lower than that seen in a control or higher or lower than that previously observed in the patient indicates that the patient would benefit from modulation of a signal transduction pathway involving KRC. Alternatively, the level of expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the

subject can be detected by assaying levels of, e.g., KRC, for example, by isolating cells from the subject and determining the level of KRC or a molecule in a signal transduction pathway involving KRC protein expression by standard methods known in the art, including Western blot analysis, immunoprecipitations, enzyme linked immunosorbent assays (ELISAs) and immunofluorescence. Antibodies for use in such assays can be made using techniques known in the art and/or as described herein for making intracellular antibodies.

In another embodiment, a change in expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject results from one or more mutations (*i.e.*, alterations from wildtype), e.g., the KRC gene and mRNA leading to one or more mutations (*i.e.*, alterations from wildtype) in the amino acid sequence of the protein. In one embodiment, the mutation(s) leads to a form of the molecule with increased activity (*e.g.*, partial or complete constitutive activity). In another embodiment, the mutation(s) leads to a form of the molecule with decreased activity (*e.g.*, partial or complete inactivity). The mutation(s) may change the level of expression of the molecule for example, increasing or decreasing the level of expression of the molecule in a subject with a disorder. Alternatively, the mutation(s) may change the regulation of the protein, for example, by modulating the interaction of the mutant protein with one or more targets *e.g.*, resulting in a form of KRC that cannot be phosphorylated or cannot interact with a KRC binding partner. Mutations in the nucleotide sequence or amino acid sequences of proteins can be determined using standard techniques for analysis of DNA or protein sequences, for example for DNA or protein sequencing, RFLP analysis, and analysis of single nucleotide or amino acid polymorphisms. For example, in one embodiment, mutations can be detected using highly sensitive PCR approaches using specific primers flanking the nucleic acid sequence of interest. In one embodiment, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *PNAS* 91:360-364). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic, DNA) from the cells of the sample, contacting the nucleic acid sample with one or more

primers which specifically amplify a sequence under conditions such that hybridization and amplification of the sequence (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In one embodiment, the complete nucleotide sequence for KRC or a molecule in a signal transduction pathway involving KRC can be determined. Particular techniques have been developed for determining actual sequences in order to study polymorphism in human genes. See, for example, Proc. Natl. Acad. Sci. U.S.A. 85, 544-548 (1988) and Nature 330, 384-386 (1987); Maxim and Gilbert. 1977. *PNAS* 74:560; Sanger 1977. *PNAS* 74:5463. In addition, any of a variety of automated sequencing procedures can be utilized when performing diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Restriction fragment length polymorphism mappings (RFLPS) are based on changes at a restriction enzyme site. In one embodiment, polymorphisms from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of a specific ribozyme cleavage site.

Another technique for detecting specific polymorphisms in particular DNA segment involves hybridizing DNA segments which are being analyzed (target DNA) with a complimentary, labeled oligonucleotide probe. See Nucl. Acids Res. 9, 879-894 (1981). Since DNA duplexes containing even a single base pair mismatch exhibit high thermal instability, the differential melting temperature can be used to distinguish target DNAs that are perfectly complimentary to the probe from target DNAs that only differ by a single nucleotide. This method has been adapted to detect the presence or absence of a specific restriction site, U.S. Pat. No. *4,683,194*. The method involves using an end-labeled oligonucleotide probe spanning a restriction site which is hybridized to a target DNA. The hybridized duplex of DNA is then incubated with the

restriction enzyme appropriate for that site. Reformed restriction sites will be cleaved by digestion in the pair of duplexes between the probe and target by using the restriction endonuclease. The specific restriction site is present in the target DNA if shortened probe molecules are detected.

Other methods for detecting polymorphisms in nucleic acid sequences include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the polymorphic sequence with potentially polymorphic RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels. See, for example, Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In another embodiment, alterations in electrophoretic mobility can be used to identify polymorphisms. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79). Single-stranded DNA fragments of sample and control nucleic acids can be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is

more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet* 7:5).

In yet another embodiment, the movement of nucleic acid molecule comprising polymorphic sequences in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA can be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting polymorphisms include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the polymorphic region is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different polymorphisms when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Another process for studying differences in DNA structure is the primer extension process which consists of hybridizing a labeled oligonucleotide primer to a template RNA or DNA and then using a DNA polymerase and deoxynucleoside triphosphates to extend the primer to the 5' end of the template. Resolution of the labeled primer extension product is then done by fractionating on the basis of size, e.g., by electrophoresis via a denaturing polyacrylamide gel. This process is often used to compare homologous DNA segments and to detect differences due to nucleotide insertion or deletion. Differences due to nucleotide substitution are not detected since size is the sole criterion used to characterize the primer extension product.

Another process exploits the fact that the incorporation of some nucleotide analogs into DNA causes an incremental shift of mobility when the DNA is

subjected to a size fractionation process, such as electrophoresis. Nucleotide analogs can be used to identify changes since they can cause an electrophoretic mobility shift. See, U.S. Pat. No. *4,879,214*.

Many other techniques for identifying and detecting polymorphisms are known to those skilled in the art, including those described in "DNA Markers: Protocols, Applications and Overview," G. Caetano-Anolles and P. Gresshoff ed., (Wiley-VCH, New York) 1997, which is incorporated herein by reference as if fully set forth.

In addition, many approaches have also been used to specifically detect SNPs. Such techniques are known in the art and many are described e.g., in DNA Markers: Protocols, Applications, and Overviews. 1997. Caetano-Anolles and Gresshoff, Eds. Wiley-VCH, New York, pp199-211 and the references contained therein). For example, in one embodiment, a solid phase approach to detecting polymorphisms such as SNPs can be used. For example an oligonucleotide ligation assay (OLA) can be used. This assay is based on the ability of DNA ligase to distinguish single nucleotide differences at positions complementary to the termini of co-terminal probing oligonucleotides (see, e.g., Nickerson et al. 1990. *Proc. Natl. Acad. Sci. USA* 87:8923. A modification of this approach, termed coupled amplification and oligonucleotide ligation (CAL) analysis, has been used for multiplexed genetic typing (see, e.g., Eggerding 1995 *PCR Methods Appl.* 4:337); Eggerding et al. 1995 Hum. Mutat. 5:153).

In another embodiment, genetic bit analysis (GBA) can be used to detect a SNP (see, e.g., Nikiforov et al. 1994. Nucleic Acids Res. 22:4167; Nikiforov et al. 1994. PCR Methods Appl. 3:285; Nikiforov et al. 1995. Anal Biochem. 227:201). In another embodiment, microchip electrophoresis can be used for high-speed SNP detection (see e.g., Schmalzing et al. 2000. *Nucleic Acids Research*, 28). In another embodiment, matrix-assisted laser desorption/ionization time-of-flight mass (MALDI TOF) mass spectrometry can be used to detect SNPs (see, e.g., Stoerker et al. Nature Biotechnology 18:1213).

In another embodiment, a difference in a biological activity of KRC between a subject and a control can be detected. For example, an activity of KRC or a molecule in a signal transduction pathway involving KRC can be detected in cells of a subject suspected of having a disorder associated with aberrant biological activity of

KRC. The activity of KRC or a molecule in a signal transduction pathway involving KRC α in cells of the subject could then be compared to a control and a difference in activity of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject as compared to the control could be used to diagnose the subject as one that would benefit from modulation of an KRC activity. Activities of KRC or molecules in a signal transduction pathway involving KRC can be detected using methods described herein or known in the art.

In preferred embodiments, the diagnostic assay is conducted on a biological sample from the subject, such as a cell sample or a tissue section (for example, a freeze-dried or fresh frozen section of tissue removed from a subject). In another embodiment, the level of expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject can be detected *in vivo*, using an appropriate imaging method, such as using a radiolabeled antibody.

In one embodiment, the level of expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the test subject may be elevated (*i.e.*, increased) relative to the control not associated with the disorder or the subject may express a constitutively active (partially or completely) form of the molecule. This elevated expression level of, e.g., KRCor expression of a constitutively active form of KRC, can be used to diagnose a subject for a disorder associated with increased KRC activity.

In another embodiment, the level of expression of KRC or a molecule in a signal transduction pathway involving KRC in cells of the subject may be reduced (*i.e.*, decreased) relative to the control not associated with the disorder or the subject may express an inactive (partially or completely) mutant form of KRC. This reduced expression level of KRC or expression of an inactive mutant form of sKRC can be used to diagnose a subject for a disorder, such as immunodeficiency disorders characterized by insufficient cytokine production.

In one embodiment, the level of expression of gene whose expression is regulated by KRC can be measured (e.g., IL-2).

In another embodiment, an assay diagnosing a subject as one that would benefit from modulation of KRC expression, post-translational modification, and/or

activity (or a molecule in a signal transduction pathway involving KRC is performed prior to treatment of the subject.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe/primer nucleic acid or other reagent (e.g., antibody), which may be conveniently used, *e.g.*, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving KRC or a molecule in a signal transduction pathway involving KRC.

VI. Kits of the Invention

Another aspect of the invention pertains to kits for carrying out the screening assays, modulatory methods or diagnostic assays of the invention. For example, a kit for carrying out a screening assay of the invention can include an indicator composition comprising KRC or a molecule in a signal transduction pathway involving KRC, means for measuring a readout (*e.g.*, protein secretion) and instructions for using the kit to identify modulators of biological effects of KRC. In another embodiment, a kit for carrying out a screening assay of the invention can include cells deficient in KRC or a molecule in a signal transduction pathway involving KRC, means for measuring the readout and instructions for using the kit to identify modulators of a biological effect of KRC.

In another embodiment, the invention provides a kit for carrying out a modulatory method of the invention. The kit can include, for example, a modulatory agent of the invention (*e.g.*, KRC inhibitory or stimulatory agent) in a suitable carrier and packaged in a suitable container with instructions for use of the modulator to modulate a biological effect of KRC.

Another aspect of the invention pertains to a kit for diagnosing a disorder associated with a biological activity of KRC in a subject. The kit can include a reagent for determining expression of KRC (*e.g.*, a nucleic acid probe for detecting KRC mRNA or an antibody for detection of KRC protein), a control to which the results of the subject are compared, and instructions for using the kit for diagnostic purposes.

VII Immunomodulatory compositions

Agents that modulate KRC activity, expression, processing, post-translational modifications, or activity, expression, processing, post-translational modification of one or more molecules in a signal transduction pathway involving KRC are also appropriate for use in immunomodulatory compositions. Stimulatory or inhibitory agents of the invention can be used to up or down regulate the immune response in a subject. In preferred embodiments, the humoral immune response is regulated.

The modulating agents of the invention can be given alone, or in combination with an antigen to which an enhanced immune response or a reduced immune response is desired.

In one embodiment, agents which are known adjuvants can be administered with the subject modulating agents. At this time, the only adjuvant widely used in humans has been alum (aluminum phosphate or aluminum hydroxide). Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants used in research and veterinary applications have potential use in human vaccines. However, new chemically defined preparations such as muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates such as those described by Goodman-Snitkoff *et al.* J. Immunol. 147:410-415 (1991) resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether, enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol can also be used. In embodiments in which antigen is administered, the antigen can *e.g.*, be encapsulated within a proteoliposome as described by Miller *et al.*, J. Exp. Med. 176:1739-1744 (1992) and incorporated by reference herein, or in lipid vesicles, such as Novasome TM lipid vesicles (Micro Vescular Systems, Inc.,Nashua, N. H.), to further enhance immune responses.

In one embodiment, a nucleic acid molecule encoding KRC (e.g., a sense or antisense or siRNA molecule or a molecule in a signal transduction pathway involving KRC or portion thereof is administered as a DNA vaccine. This can be done using a plasmid DNA construct which is similar to those used for delivery of reporter or

therapeutic genes. Such a construct preferably comprises a bacterial origin of replication that allows amplification of large quantities of the plasmid DNA; a prokaryotic selectable marker gene; a nucleic acid sequence encoding, e.g., a KRC polypeptide or portion thereof; eukaryotic transcription regulatory elements to direct gene expression in the host cell; and a polyadenylation sequence to ensure appropriate termination of the expressed mRNA (Davis. 1997. *Curr. Opin. Biotechnol.* 8:635). Vectors used for DNA immunization may optionally comprise a signal sequence (Michel *et al.* 1995. *Proc. Natl. Acad. Sci USA.* 92:5307; Donnelly *et al.* 1996. *J. Infect Dis.* 173:314). DNA vaccines can be administered by a variety of means, for example, by injection (*e.g.*, intramuscular, intradermal, or the biolistic injection of DNA-coated gold particles into the epidermis with a gene gun that uses a particle accelerator or a compressed gas to inject the particles into the skin (Haynes *et al.* 1996. *J. Biotechnol.* 44:37)). Alternatively, DNA vaccines can be administered by non-invasive means. For example, pure or lipid-formulated DNA can be delivered to the respiratory system or targeted elsewhere, *e.g.*, Peyers patches by oral delivery of DNA (Schubbert. 1997. *Proc. Natl. Acad. Sci. USA* 94:961). Attenuated microorganisms can be used for delivery to mucosal surfaces. (Sizemore *et al.* 1995. *Science.* 270:29)

In one embodiment, plasmids for DNA vaccination can express KRC (or antagonist of KRC as well as the antigen against which the immune response is desired or can encode modulators of immune responses such as lymphokine genes or costimulatory molecules (Iwasaki *et al.* 1997. *J. Immunol.* 158:4591).

VIII. Administration of KRC Modulating Agents

KRC modulating agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to either enhance or suppress immune responses (*e.g.*, T cell mediated immune responses). By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the modulating agent. The term subject is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof, including but not

limited to the transgenic KRC mouse described herein. Administration of an agent as described herein can be in any pharmacological form including a therapeutically active amount of an agent alone or in combination with a pharmaceutically acceptable carrier.

Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a KRC modulating agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of peptide to elicit a desired response in the individual. Dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including for example intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in ex vivo treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF). When it is intended that a KRC modulator be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of KRC polypeptide across the blood-brain barrier.

The KRC modulator can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, KRC can be coupled to any substance known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor, and administered by intravenous injection. (See for example, Friden *et al.*, 1993, *Science* 259: 373-377 which is incorporated by reference). Furthermore, KRC can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See for example Davis *et al.*, 1978, *Enzyme Eng* 4: 169-73; Burnham, 1994, *Am J Hosp Pharm* 51: 210-218, which are incorporated by

reference).

Furthermore, the KRC modulator can be in a composition which aids in delivery into the cytosol of a cell. For example, the agent may be conjugated with a carrier moiety such as a liposome that is capable of delivering the peptide into the cytosol of a cell. Such methods are well known in the art (for example see Amselem *et al.*, 1993, *Chem Phys Lipids* 64: 219-237, which is incorporated by reference). Alternatively, the KRC modulator can be modified to include specific transit peptides or fused to such transit peptides which are capable of delivering the KRC modulator into a cell. In addition, the agent can be delivered directly into a cell by microinjection.

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. KRC can also be incorporated into a solid or semi-solid biologically compatible matrix which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral

administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used. It is also provided that certain formulations containing the KRC modulator are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, olyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals. The specific dose can be readily calculated by one of ordinary skill in the art, *e.g.*, according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for

treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in assay preparations of target cells. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method for the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In one embodiment of this invention, a KRC modulator may be therapeutically administered by implanting into patients vectors or cells capable of producing a biologically-active form of KRC or a precursor of KRC, *i.e.* a molecule that can be readily converted to a biological-active form of KRC by the body. In one approach cells that secrete KRC may be encapsulated into semipermeable membranes for implantation into a patient. The cells can be cells that normally express KRC or a precursor thereof or the cells can be transformed to express KRC or a biologically active fragment thereof or a precursor thereof. It is preferred that the cell be of human origin and that the KRC polypeptide be human KRC when the patient is human. However, the formulations and methods herein can be used for veterinary as well as human applications and the term "patient" or "subject" as used herein is intended to include human and veterinary patients.

Monitoring the influence of agents (*e.g.*, drugs or compounds) on the expression or activity of a KRC protein can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase KRC gene expression, protein levels; or upregulate KRC activity, can be monitored in clinical trials of subjects exhibiting decreased KRC gene expression, protein levels, or downregulated KRC activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease KRC gene expression, protein levels, or downregulate KRC activity, can be monitored in clinical trials of subjects exhibiting increased KRC gene expression, protein levels, or upregulated KRC activity. In such clinical trials, the expression or activity of a KRC gene, and preferably, other genes that have been implicated in a disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including KRC, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates KRC activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on a KRC associated disorder, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of KRC and other genes implicated in the KRC associated disorder, respectively. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by

measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of KRC or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a KRC protein, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the KRC protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the KRC protein, mRNA, or genomic DNA in the pre-administration sample with the KRC protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of KRC to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of KRC to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an embodiment, KRC expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

In a preferred embodiment, the ability of a KRC modulating agent to modulate inflammation or apoptosis in a epithelial cell of a subject that would benefit from modulation of the expression and/or activity of KRC can be measured by detecting an improvement in the condition of the patient after the administration of the agent. Such improvement can be readily measured by one of ordinary skill in the art using indicators appropriate for the specific condition of the patient. Monitoring the response of the patient by measuring changes in the condition of the patient is preferred in

situations were the collection of biopsy materials would pose an increased risk and/or detriment to the patient.

It is likely that the level of KRC may be altered in a variety of conditions and that quantification of KRC levels would provide clinically useful information. Furthermore, because it has been demonstrated herein that increased levels of KRC expressed by a cell can shift the cell death regulatory mechanism of that cell to decrease viability, it is believed that measurement of the level of KRC in a cell or cells such as in a group of cells, tissue or neoplasia, like will provide useful information regarding apoptotic state of that cell or cells. In addition, it can also be desirable to determine the cellular levels of these KRC-interacting polypeptides.

Furthermore, in the treatment of disease conditions, compositions containing KRC can be administered exogenously and it would likely be desirable to achieve certain target levels of KRC polypeptide in sera, in any desired tissue compartment or in the affected tissue. It would, therefore, be advantageous to be able to monitor the levels of KRC polypeptide in a patient or in a biological sample including a tissue biopsy sample obtained form a patient and, in some cases, also monitoring the levels of KRC and, in some circumstances, also monitoring levels of TRAF, c-Jun or another KRC-interacting polypeptide. Accordingly, the present invention also provides methods for detecting the presence of KRC in a sample from a patient.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis *et al.* U.S. Patent NO: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular

Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu *et al.* eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the figures and the sequence listing, are hereby incorporated by reference.

EXAMPLES

The following materials and methods were used throughout the Examples:

Cell Lines, Plasmids and Stable and Transient Transfection Assays

The human embryonic kidney cell line HEK293, the NIH/3T3 fibroblast cells and the macrophage cell line RAW were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HEK293 cells (4 X 510 5 per well) were seeded in 6 well plates, and 12 h later cells were transfected with EFECTENE™ (Qiagen) with 25 ng of a 2XNFκB-luciferase (Luc) reporter gene plasmid and 0.5 μg of the indicated TRAF and KRC expression vectors. Total amounts of transfected DNA were kept constant by supplementing with control empty expression vector plasmids as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined via the luciferase assay system (PROMEGA). PRSV-βGal vector (50 ng) was used to normalize for transfection efficiency by measuring β galactosidase activity using the Galacton-PLUS substrate system (TROPIX, Inc.). Whenever indicated, the cells were treated for 4 hours with TNFα or IL-1 (10ng/ml). To generate stable transfectants, EFECTENE™ mediated

transfection of the RAW cell line was performed and clones were selected and maintained in complete medium supplemented with G418 (2 mg/ml).

Yeast Two Hybrid Screen

The yeast strain EGY48, containing the reporter genes for LEU and β–galactosidase activity under the control of an upstream LexA-binding site was used as a host for the two hybrid screen. The KRC fragment from amino acid 204 to 1055 (KRC tr) (*Figure 2*(A)) was fused in frame to the LexA DNA binding domain and a yeast strain expressing the LexA-KRC tr fusion protein was transfected with a mouse Th1 clone cDNA library (Szabo, *et al.*) fused to the GAL4 transcriptional activation domain. Transformants were plated on agar selection media lacking uracil, tryptophan, leucine and histidine. The resulting colonies were isolated and retested for growth in Leu^- plates and for β galactosidase activity. Plasmid DNA was purified from colonies that were $Leu^+\beta gal^+$ and used for retransformation of a yeast strain expressing a heterologous bait to determine the specificity of interaction.

Northern Blot Analysis

Total RNA was isolated from transfected RAW macrophage cells using TRIZOL™ reagent (Gibco/BRL) and 15 μg of each sample separated on 0.8% agarose 6% formaldehyde gels, transferred onto GeneScreen™ membrane (NEN) in 20X SSC overnight and covalently bound using a UV Stratalinker™ (Stratagene). Hybridization of blots was carried out at 42°C as described (Hodge, *et al.*) using the radiolabeled TNFα, KRC (5850-6210) and HPRT probes prepared with the Random primer kit (Boehringer Mannheim).

Western Blot Analysis

Effectene™ mediated transfections into 293T cells were performed. To prepare cell extracts, cells were washed twice with PBS and lysed for 10 minutes on ice in 1 ml Triton lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 2mM DTT and complete protease inhibitor mixture (Roche Molecular Biochemicals), and the lysates were cleared by centrifugation for 10 min at 14 000 rpm. The cell lysates were precleared with 30 μl of protein A/G-Sepharose beads

and then incubated for 4h with 25 µl of anti-MYC antibody directly conjugated to sepharose beads. The immunoprecipitates were then washed 5 times with the lysis buffer, resuspended in SDS sample buffer, and heated at 95^0 C for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitocellulose membrane (Schleicher & Schuell) and western blotting performed by probing with primary antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

In vitro Kinase Assay

Anti-HA or anti-FLAG immunoprecipitates were used for immune complex kinase assays that were performed at 30^0 C for 30 min with 1 µg of substrate, 10 µCi of γ^{32} P ATP, and 10 µM ATP in 30 µl of kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 25 mM β-glycerophosphate, 50 µm NA3VO4, and 50 µm DTT). The substrate was GST-c-JUN.

Apoptosis Assay

β–galactosidase cotransfection assays for determination of cell death were performed as described (Hsu, *et al.*). Transfected NIH 3T3 cells were washed with PBS, fixed in PBS containing 3% paraformaldehyde for 10 min at 4^0 C, and washed with PBS. Fixed cells were stained overnight with XGal. The number of blue-stained cells was determined microscopically. The average number from one representative experiment of three is shown.

Luciferase Assays

For each transfection, 5 x 10^6 Jurkat cells were incubated with either IL2-Luc, NFAT/AP1-Luc or AP1-Luc reporter DNA together with pEF vector or pEF-KRC and CMV-βGAL as normalization control in 0.4 ml of RPMI and transfected by electroporation (260 v, 975uF). Transfected cells were cultured at 37° C for 20 h in RPMI 1640 medium (Gibco BRL) supplemented with 10 % fetal bovine serum. Transfected cells were stimulated with PMA (50ng/ml) and ionomycin (2uM) for 6

hours prior to luciferase (Promega) and β-galactosidase assays (Galacton-PLUS substrate system, TROPIX, Inc).

Reverse Transcription-PCR

Total RNA was isolated from T cells using TRIZOL™ reagent (Gibco/BRL). One (1) μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad). PCR was performed with 2uM of each primer (listed below) and 2.5 units of Platinum High fidelity enzyme (Invitrogen) according to the manufacturer.
IL2F 5'CAAGAATCCAAACTCACCAG3' (SEQ ID NO:3),
IL2R 5'TAGCAACCATACATTCAACAA3' (SEQ ID NO:4)
KRCF 5'CTCCAATACAGAATTCAAGGGC3' (SEQ ID NO:5),
KRCR5'TTTAGGTTGGCCAGTGTGTGTG (SEQ ID NO:6)

Jurkat Cell Activation With Raji B Lymphoma Cells and Staphylococcal Enteroxin E (SEE)

Jurkat cells were transfected by electroporation and incubated for 20 h at 37^0 C before stimulation for 8 h with the Raji B cell line and Staphylococcal Enteroxin E (SEE) using Raji cells (1:1 with Jurkat cells) and SEE (200 ng/ml).

Pull Down Assays

In vitro translated c-Jun (35^S methionine labeled) and His-KRCtr were incubated for 2h at 4^0 C in binding buffer (PBS/0.25 % Nonidet p-40/1mM PMSF/0.25 mM DTT), incubated for 2 hours with the anti-HIS antibody (Santa Cruz), 30 μl of protein A/G sepharose added and the reaction incubated at 4^0 C for an additional 2h. The immunoprecipitates were then washed five times with the binding buffer, resuspended in SDS sample buffer, and heated at 95^0 C for 5 min.

Retroviral Gene Transduction

Activated $CD4^+$T cells were transduced by RV, RV-KRC or RV-ZAS2 as described previously (Szabo, S.J., *et al.* (2000) *Cell* 100:655-669).

Generation of KRC-Deficient Mice and Subsequent T Cell Stimulation

ES cells were generated in which the entire 5.4 kB exon 2 of KRC was replaced by a neomycin cassette resulting in an allele that produces no KRC protein. KRC +/- ES cells transmitted the disrupted allele to 129/B6 offspring. Heterozygous pups were backcrossed to wild type B6 mice. Mice analyzed were progeny of intercrosses between heterozygous F3 generation backcrossed 129/B6 mice. CD4+ T cells were purified by positive selection from spleen and lymph nodes of 6-8 week old male KRC +/+ and KRC -/- littermates using magnetic beads according to the instructions of the manufacturer (Miltenyi Biotec). Cells were stimulated at 10^6 cells/mL with plate-bound anti-CD3 (1.0 μg/mL) plus anti-CD28 (0.5 μg/mL). Twenty-four hours later, supernatants were collected and analyzed for IL-2 levels by ELISA. Additionally, cells were stimulated for 72 hours in the presence of 200 U/mL human IL-2, and supernatants were collected and analyzed for IFNγ levels by ELISA.

EXAMPLE 1: INTERACTION OF KRC WITH TRAF FAMILY MEMBERS IN YEAST

(A) In this example, a yeast two-hybrid interaction trap was used to select a T cell cDNA library for sequences encoding polypeptides that specifically interacted with a KRC-LexA fusion protein. As bait KRC sequences encoding amino acids 204 to 1055 (KRC tr) were used which include the third zinc finger domain, one of the three acidic domains and the putative NLS sequence, expressed in the pEG202 vector (*Figure 1(A)*). One class of interactors encoding a fusion protein with apparently high affinity for the KRC-LexA bait as exhibited by high level of β-galactosidase activity and ability to confer leucine prototrophy was isolated and upon sequencing proved to be the C-terminal segment of TRAF1. The interaction with TRAF1 was specific since no interaction was detected with control plasmids that encode KRC, c-Maf or relA fusion proteins or with the control vector alone

(B) The ability of TRAF proteins to interact specifically with KRC *in vivo* was tested in mammalian cells. KRC sequences 204-1055 were subcloned into a mammalian expression vector which fuses the coding region to an N-terminal epitope tag from a myc peptide, and the expression of the protein confirmed by direct western

blot analysis with anti-MYC antibody (*Figure 1(B)*, right panel). This tagged construct was then cotransfected with TRAF-FLAG-tagged expression plasmids into 293T cells and lysates prepared for immunoprecipitation with an anti-MYC antibody. A STAT4-FLAG-tagged expression construct was used as negative control.

Western blot analysis of these samples using an anti-FLAG-specific monoclonal antibody (mAb) demonstrated that the anti-MYC antibody coimmunoprecipitated all six FLAG-tagged TRAFs, but not the STAT4 control protein (*Figure 1(B)*, left panel). Finally, the deletion of the ring finger of TRAF2 (TRAF2 DN) did not alter its interaction with KRC (*Figure 1(D)*), consistent with our isolation of TRAF1, which lacks the RING finger, in the yeast two hybrid interaction trap screen. These results demonstrate that KRC does interact with all TRAF family members and that this interaction is likely occurring through the TRAF C domain.

(C) Coimmunoprecipitation assays in the presence of more stringent, higher salt conditions were performed. As shown in *Figure 1(C)*, when 300mM rather than 137mM NaCl was used, TRAF5 was not able to coimmunoprecipitate with KRC, and the amount of TRAFs 3, 4 and 6 that could be immunoprecipitated was reduced. The TRAF-C domain of TRAF1 and TRAF2 share 70 % identity but share less than 43% identity with TRAF5 and TRAF(D) To further explore if KRC interacted with a higher affinity with TRAF1 and TRAF2 and with lower affinity with the other TRAF members, we tested the association of endogenous rather than overexpressed TRAFs with ectopically expressed KRC. 293T cells (which lack TRAF1) were transfected with plasmids encoding MYC-tagged KRC or empty vector and 24 hours after transfection cells were lysed. Lysates from 293T cells were incubated with anti-MYC antibody to precipitate KRC.

Subsequent Western blotting with anti-TRAF2, anti-TRAF5 or anti-TRAF6 mAbs showed that only endogenous TRAF2 was able to interact with over-expressed KRC (*Figure 1(E)*). The bands observed in the TRAFs 5 and 6 coimmunoprecipitants are non-specific Furthermore, treatment of 293T cells with TNF or IL-1 to induce TRAF activity did not affect the strength of the interaction between TRAF2 and ectopically expressed KRC).

Taken together, these data demonstrate that KRC interacts with TRAF family members, that this interaction occurs through the TRAF-C domain, and that KRC interacts with higher affinity with TRAF2 than with TRAF5 and TRAF6. This result is consistent with the higher sequence conservation between the TRAF domain of TRAF1 and TRAF2 than between the other TRAF family members.

EXAMPLE 2: KRC PREVENTS TRAF DEPENDENT NFκB ACTIVATION

In this example, the effect of KRC overexpression on TRAF2, TRAF5 and TRAF6-induced NFκB dependent gene expression using transfection assays in 293T human embryonic kidney cells was tested. The results show that overexpression of both the full-length KRC and the KRC 204-1055 (KRC truncated, tr) in the absence of exogenous TRAFs blocked NFκB-dependent transactivation in a manner comparable in strength to the inhibition observed with a dominant negative form of TRAF2 (*Figure 2(A)*). The results also show that both the KRC tr and the full length KRC blocked TRAF2-induced NFκB activation (*Figure 2(B)*) while NFκB activation induced by TRAF5 and TRAF6 were substantially but not completely affected (*Figures 2(C) and 2(D)*).

EXAMPLE 3: ANTISENSE AND DOMINANT NEGATIVE KRC INCREASE CYTOKINE DRIVEN NFκB TRANSACTIVATION WHILE SENSE KRC IS INHIBITORY.

(A) In this example, whether KRC overexpression affects TNFα-induced NFκB transactivation in 293 cells was tested. *Figure 3* shows that overexpression of KRC or KRC tr in 293 cells strongly inhibited TNFα-induced NFκB activation to a level comparable with the TRAF2 DN effect in the presence of TNFα (*Figure 3(A)*). These data are consistent with the demonstrated effect of TRAF2 on NFκB-dependent gene activation in certain cell types, *e.g.,* B cells, as shown in TRAF2-deficient mice (Yeh, *et al.*).

(B) To manipulate the endogenous KRC, an antisense KRC construct (H10AS) and a dominant negative construct expressing only the ZAS2 domain of KRC (ZAS2) was used (*Figure 1(A)*). Both the antisense and the ZAS2 expressing constructs greatly enhanced transactivation of the NFκB reporter upon induction with TNFα

(*Figure 3(B)*). The same results were obtained with the antisense KRC (*Figure 3(C)*) and dominant negative KRC when NFκB-dependent transactivation was driven by exogenous TRAF2 overexpression. These results demonstrate that KRC under normal conditions behaves as a negative regulator of TRAF2-mediated NFκB activation.

EXAMPLE 4: IKKβ OVEREXPRESSION OVERCOMES KRC INHIBITION OF NFκB-DEPENDENT TRANSACTIVATION

In this example, whether KRC affected NFκB-driven gene activation by interfering with upstream events was tested. Full-length KRC or KRC tr, and as a control, the TRAF2 DN mutant, were overexpressed in 293 cells in the absence or presence of ectopic IKKβ (IκB kinase) and the effect on NFκB-mediated transactivation determined. The activation of IKKβ is a key step in the nuclear translocation of the transcription factor NF-κB. IKK is a complex composed of three subunits: IKKα, IKKβ, and IKKγ (also called NEMO). In response to the proinflammatory cytokine tumor necrosis factor (TNF), IKK is activated after being recruited to the TNF receptor 1 (TNF-R1) complex via TNF receptor-associated factor 2 (TRAF2). *Figure 4* demonstrates that overexpression of IKKβ overcomes the inhibitory effect of both KRC and KRC tr in a manner comparable to its effect on TRAF2 DN. Since IKK activation is downstream of TRAF activation, these results demonstrate that the effect of KRC on NFκB-driven gene expression is due to its ability to interact with TRAFs rather than to competition with NFκB for binding to DNA.

EXAMPLE 5: KRC INCREASES TNFα-INDUCED APOPTOSIS

In this example, whether KRC is involved in the apoptotic process was tested. KRC was overexpressed in 3T3 cells apoptosis was measured by counting β-galactosidase positive (live) cells (*Figure 5*). As previously described for HeLa cells, these results demonstrate that in 3T3 cells apoptosis can be induced when either IκB DN or TRAF2 DN are overexpressed in the presence of TNFα, but cannot be induced by TNFα alone (Hsu, *et al.*; Hsu, *et al.*; Liu, *et al.*). KRC overexpression resulted in an increase in TNF mediated cytotoxicity equivalent to that observed with overexpression of IκB or TRAF2 DN. The same effect was observed with the KRC tr construct

indicating that KRC likely sensitizes cells to TNFα-induced death by inhibiting NFκB induction, most probably through its effect on blocking TRAF2 function. Collectively, these results demonstrate that upon TNF receptor activation, the NFkB, TRAF1, TRAF2, c-IAP-1 and c-IAP-2 pathways operate as a positive feedback system to amplify the survival signal to protect cells from TNF-induced injury. The interaction of KRC with TRAF2, and possibly with TRAF1 in other cell types, acts to inhibit TRAF activity thereby balance between pro-apoptotic and anti-apoptotic stimuli.

EXAMPLE 6: KRC PREVENTS TRAF2 AND TNFα-DEPENDENT JNK ACTIVATION

In this example, whether KRC could block TRAF2 dependent JNK activation was tested. The KRC 204-1055 tr construct, full length KRC, ZAS2 expressing construct and the antisense KRC were cotransfected into 293 cells together with TRAF2, and JNK activity measured 24 hours after transfection. Both the KRC tr and the full length KRC blocked TRAF2-dependent JNK activation (*Figure 6(A)*). Full length KRC blocked JNK activation only partially, likely due to the approximately 10 fold lower expression of this construct as compared to KRC tr. The results also show a dramatic increase of TRAF2 dependent JNK activation with expression of both the antisense KRC as well with the dominant negative ZAS2 expressing construct. The same results were obtained when JNK activation was induced by treatment with TNFα (*Figure 6(B)*). A careful time course of JNK activation was performed, mediated by TNFα in the presence of antisense KRC, which revealed sustained JNK activation as compared to control vector alone (*Figure 6(C)*). These results demonstrate that KRC negatively modulates JNK activation by inhibiting TRAF2 function. The immediate target of TRAF2 in TNF-induced JNK/SAPK activation may be the MAP3 kinase ASK1 or members of the GCK family of kinases.

EXAMPLE 7: KRC IS A NEGATIVE REGULATOR OF ENDOGENOUS TNFα EXPRESSION

In this example, whether KRC can modulate the expression of endogenous TNFα was tested. Overexpressed KRC or dominant negative KRC was transfected in the RAW macrophage cell line and levels of TNFα in a panel of

transfectant clones were analyzed. RAW transfectants stably overexpressing KRC displayed a substantial decrease of baseline TNFα mRNA transcripts when compared to control vector transfected RAW cells while RAW transfectants expressing the dominant negative version had substantial increase in TNFα expression (*Figure 7*). These results demonstrate that KRC acts to inhibit the transcription of the TNFα proinflammatory cytokine and that this may occur both through its inhibition of NFκB and JNK signaling pathways.

EXAMPLE 8: KRC TRANSLOCATES FROM CYTOSOL TO NUCLEUS UPON CELL ATTACHMENT

In this example, how KRC (originally decried as a nuclear protein) physiologically interacts with the predominantly cytosolic TRAF2 to affect gene activation was tested. A full-length KRC was fused to GFP and its cellular localization upon transfection into 3T3 cells was examined. In 3T3 cells in suspension, KRC was mainly localized to the cytosol while in 3T3 cells that had adhered to the glass slide, KRC was primarily present in the nucleus (*Figure 8*). These results clearly demonstrate that KRC can reside in the cytosol where it can interact with TRAF2. It should be noted that TRAF2 has recently been described to translocate from cytosol to nucleus as well (Min, *et al*, 1998). Thus KRC and TRAF2 may well interact in both subcellular compartments.

EXAMPLE 9: KRC IS TH1 SPECIFIC

In this example, KRC expression in primary T cells was measured. RT-PCR analysis of KRC expression in primary T cells was performed. KRC expression was measured at 24 hours and 72 hours. The results demonstrate that KRC expression is rapidly lost in Th2 cells at 72 hours whereas KRC expression in Th1 cells is maintained at 72 hours (*Figure 9*). These results demonstrate that KRC is Th1 specific.

EXAMPLE 10: KRC ACTIVATES T CELLS

In this example, KRC was transfected into Jurkat T cells and CD69 expression was measured by FACS analysis. The results show that KRC overexpression increases expression of CD69 (a T cell activation marker) in Jurkat T cells (*Figure 10*).

EXAMPLE 11: KRC INCREASES IL-2 GENE TRANSCRIPTION IN THE PRESENCE OF PMA/IONOMYCIN

This example shows that KRC increases IL-2 gene transcription in the presence of PMA/Ionomycin. This increase in IL-2 transcription occurs primarily through activating AP-1 with no contribution from NFAT. *Figure 11(A)* shows IL-2 promoter transactivation by KRC in Jurkat T cells activated by PMA/Ionomycin. *Figure 11(B)* shows transactivation of a composite NFAT-AP1 reporter by KRC. *Figure 11(C)* shows transactivation of an AP-1 reporter by KRC.

EXAMPLE 12: KRC INCREASES IL-2 GENE TRANSCRIPTION IN THE PRESENCE OF B CELL ANTIGEN PRESENTING CELLS

In this example, the results demonstrate that KRC increases IL-2 gene transcription in the presence of B cell antigen presenting cells and superantigen SEE and does so primarily through activating AP-1 with no contribution from NFAT. *Figure 12(A)* shows IL-2 promoter transactivation by KRC in Jurkat T cells activated by the Raji B cell APC line and the superantigen SEE. *Figure 12(B)* shows transactivation of a composite NFAT-AP1 reporter by KRC. *Figure 12(C)* shows transactivation of an AP-1 reporter by KRC.

EXAMPLE 13: KRC OVEREXPRESSION INCREASES ENDOGENOUS IL-2 PRODUCTION WHILE KRC LOSS DECREASES ENDOGENOUS IL-2 PRODUCTION

In this example, increased IL-2 production in Jurkat T cells stably expressing KRC was measured by ELISA. IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by an antigen presenting cell (Jain, J., *et al.* (1995) *Curr. Biol.* 7:333-342). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T cell activation in the human T cell lymphoma Jurkat. To assess the function of KRC in T cells, Jurkat cells, which express barely detectable levels of endogenous KRC protein by Western blot analysis, were stably transfected with a plasmid encoding full-length KRC (pEF-KRC) or with vector only

control (pEF). G418 drug- resistant Jurkat clones were expanded and analyzed for IL-2 secretion following activation. Clones stably expressing KRC showed clear increases in KRC protein levels, as detected by Western blotting All clones expressing pEF-KRC produced substantially greater amounts of IL-2 upon PMA and ionomycin treatment than activated Jurkat clones transfected with the control vector (*Figure 13(A)*). KRC overexpression alone was not sufficient to induce IL-2 secretion, as no IL-2 was detected in the culture supernatants of unstimulated KRC-overexpressing clones These results suggested that KRC is able to boost IL-2 secretion in concert with signals emanating from the TCR.

Although the Jurkat model has proved valuable to dissect pathways of T cell signaling, certain observations made in Jurkat cells are irreproducible in primary T cells (Dumitru, C.D. *et al.* (2000) *Cell* 103:1071-1083; Weiss, L., *et al.* (2000) *J. Exp. Med.* 191: 139-145). Therefore, the effects of KRC overexpression were studied in primary CD4+T cells as well as in the Jurkat line using a retroviral delivery system to express KRC in primary CD4+T cells. Bicistronic retroviral vectors encoding full-length KRC and control GFP were generated. The KRC ZAS2 domain was previously shown to act as a dominant negative in the context of KRC mediated inhibition of TNF-induced NF-κB activation (Oukka, M., *et al.* (2002) *Mol. Cell* 9:121-131). Purified CD4+T cells were infected with these retroviruses 36 hours after primary activation with both anti-CD3 and anti-CD28, and sorted by flow cytometry for GFP expression 24 hours after infection. The ability of each population to produce IL-2 following subsequent activation by anti CD3 or anti CD3 plus CD28 was measured at 24 hours post-stimulation. As shown in *Figure 13 (B)*, CD4 cells transduced with full-length KRC produced higher amounts (approximately 3 to 4 fold increase) of IL-2 than CD4 cells infected with the GFP control retrovirus. Furthermore, CD4 cells transduced with the dominant negative KRC ZAS2 domain construct produced significantly less IL-2 than both the full-length KRC and GFP control transduced cells. These data are consistent with the notion that the ZAS2 domain interferes with endogenous KRC activity in T cells to prevent optimal expression of IL-2.

EXAMPLE 14: KRC TRANSACTIVATION OF AP-1 DEPENDS ON RAS, RAF AND PKC-THETA

In this example, the results demonstrate that KRC transactivation of AP-1 response element depends on Ras, Raf and PKC-theta signaling molecules. *Figure 14(A)* shows KRC transactivation of the AP-1 reporter is blocked by dominant negative Ras and Raf. *Figure 14(B)* shows KRC transactivation of the AP-1 reporter is blocked by dominant negative PKC-theta and by the specific PKC-theta inhibitor Rottlerin.

EXAMPLE 15: KRC CONTROLS IL-2 EXPRESSION

In this example, the results demonstrate that KRC controls IL-2 expression. RT-PCR of KRC transfected Jurkat clones was performed. The results show increased IL-2 expression upon KRC transfection (*Figure 15).*

EXAMPLE 16: KRC INCREASES ACTIN POLYMERIZATION

In this example, the results demonstrate that KRC increases actin polymerization. Immunofluorescence of F-actin upon KRC overexpression in Jurkat T cells was performed. The results show the reorganization of F-actin filaments in KRC transfected Jurkat T cells (*Figure 16).*

EXAMPLE 17: KRC EXPRESSION INCREASES IN $CD4^+$ CELLS UPON ACTIVATION

In this example, the results demonstrate that KRC expression increases in $CD4^+$ cells upon activation with anti-CD3 ((2.0 μg/mL)/anti-CD28 (1.0 μg/mL) antibodies. RT-PCR analysis demonstrates that KRC expression was induced with very rapid kinetics (within 20 minutes) in $CD4^+$ T cells upon activation and increased levels of KRC transcripts were observed throughout the duration of primary CD3/CD28 stimulation, up to 48 hours(*Figure 17).*

EXAMPLE 18: KRC OVEREXPRESSION INCREASES WHILE KRC LOSS DECREASES ENDOGENOUS IL-2 PRODUCTION IN BOTH TRANSFORMED AND PRIMARY T CELLS

IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by an antigen presenting cell (Jain, J., C. Loh, and A. Rao. 1995. 7:333-342.). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T cell activation in the human T cell lymphoma Jurkat. To assess the function of KRC in T cells, Jurkat cells, which express barely detectable levels of endogenous KRC protein by Western blot analysis, were stably transfected with a plasmid encoding full-length KRC (pEF-KRC) or with vector only control (pEF). G418 drug- resistant Jurkat clones were expanded and analyzed for IL-2 secretion following activation. Clones stably expressing KRC showed clear increases in KRC protein levels, as detected by Western blotting. All clones expressing pEF-KRC produced substantially greater amounts of IL-2 upon PMA and ionomycin treatment than activated Jurkat clones transfected with the control vector (Fig 18A). KRC overexpression alone was not sufficient to induce IL-2 secretion, as no IL-2 was detected in the culture supernatants of unstimulated KRC-overexpressing clones. These results suggested that KRC is able to boost IL-2 secretion in concert with signals emanating from the TCR.

Although the Jurkat model has proved valuable to dissect pathways of T cell signaling, certain observations made in Jurkat cells are irreproducible in primary T cells Although the Jurkat model has proved valuable to dissect pathways of T cell activation and signaling, some observations made in Jurkat cells have not been reproduced in primary T cells(Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.-H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, and P.N. Tsichlis. 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103:1071-1083, Weiss, L. et al. 2000. *J Exp Med* 191: 139-145). Therefore, the effects of KRC overexpression in primary CD4 T cells as well as in the Jurkat line were studied using a retroviral delivery system was used to express KRC in primary CD4 T cells. Bicistronic retroviral vectors encoding full-length KRC were generated, the KRC ZAS2 domain which we have previously shown acts as a dominant negative in the context of KRC mediated inhibition of TNF-induced NF-κB activation (Oukka, .NET al. 2002. *Mol. Cell* 9:121-131), and control GFP. Purified

CD4 T cells were infected with these retroviruses 36 hours after primary activation with both anti-CD3 and anti-CD28, and sorted by flow cytometry for GFP expression 24 hours after infection. The ability of each population to produce IL-2 following subsequent activation by anti CD3 or anti CD3 plus CD28 was measured at 24 hours post-stimulation. As shown in Fig 18B, CD4 cells transduced with full-length KRC produced higher amounts (approximately 3 to 4 fold increase) of IL-2 than CD4 cells infected with the GFP control retrovirus. Furthermore, CD4 cells transduced with the dominant negative KRC ZAS2 domain construct produced significantly less IL-2 than both the full-length KRC and GFP control transduced cells. These data are consistent with the notion that the ZAS2 domain interferes with endogenous KRC activity in T cells to prevent optimal expression of IL-2.

To further analyze the role of KRC in regulating endogenous IL-2 expression, CD4 cells purified from KRC-deficient mice were analyzed. Briefly, lymphoid development in these mice appears normal, with normal numbers of $CD4^+$ T cells isolated from spleen and lymph nodes. Additionally, resting CD4 cells recovered appeared phenotypically normal based on expression of maturation markers such as CD4, CD62L, CD25, CD69 and TCRß. As shown in figure 18C, KRC -/- CD4 cells activated *in vitro* for 24 hours by CD3/CD28 stimulation produced 10-fold less IL-2 production was detected than in CD4 cells from wild type littermates. However, IFNγ production by these cells following 72 hours of primary stimulation in the presence of excess exogenous IL-2 was normal (fig. 18D), suggesting that the deficiency of KRC in these cells does not globally inhibit activation-induced cytokine production. Thus, KRC is a positive regulator of IL-2 production both in Jurkat cells and, more importantly, in primary CD4 T cells.

EXAMPLE 19: KRC OVEREXPRESSION INCREASES THE TRANSCRIPTION OF THE IL-2 GENE THROUGH AN AP-1-SITE-DEPENDENT MECHANISM

In this example, the results demonstrate that KRC overexpression increases the transcription of the IL-2 gene through an AP-1-site-dependent mechanism.

The production of IL-2 by T cells is regulated at multiple levels including transcription, mRNA stability and rate of protein secretion (Lindsten, T., *et al.* (1989) *Science* 244:339; Jain, J., *et al.* (1992) *Nature* 356:801-804). In order to define at which stage(s) KRC acts, levels of IL-2 mRNA transcripts were measured by semi-quantitative RT PCR in Jurkat T cells stably transfected with full-length KRC. As seen in *Figure 19(A)*, Jurkat clones over-expressing KRC displayed higher levels of IL-2 transcripts when activated than Jurkat clones transfected with vector control. Next the ability of KRC to directly transactivate a 1.5 kb IL-2 promoter-luciferase reporter in Jurkat cells was tested. Provision of KRC resulted in an approximately 10 fold induction of luciferase activity in Jurkat cells treated with PMA plus ionomycin (*Figure 19(B)*, upper panel). Just as KRC overexpression alone did not lead to spontaneous production of endogenous IL-2, no transactivation by KRC was observed in the absence of PMA/ionomycin in these luciferase reporter assays. In order to provide a more physiologic signal to activate Jurkat cells, a model system in which Raji B lymphoma cells act as antigen presenting cells to present staphylococcal enteroxin E (SEE) to Jurkat was utilized. As shown in *Figure 19 (B)*, lower panel, provision of KRC substantially increased (approximately 10 fold) IL-2 promoter activity in this system. Interestingly, KRC had no effect on IL-2 promoter activity in the absence of Jurkat activation either by PMA/ionomycin or by antigen/APC. These data further suggest that KRC expression alone is not sufficient to induce IL-2 mRNA expression; instead, KRC's ability to enhance IL-2 production relies on endogenous factors found only in activated T cells.

KRC was originally cloned as a transcription factor, however, its effect on gene activation could clearly be ascribed to its function as an adapter protein. Nevertheless, KRC has been shown to bind both NFκB and RSS target sites *in vitro* and an NFκB site is present in the IL-2 promoter that has been shown to bind the NFκB family member c-Rel (Himes, S.R., *et al.* (1996) *Immunity* 5:479-489). To test whether KRC overexpression leads to enhanced function of a specific site in the IL-2 promoter

and to identify the site, Jurkat cells were cotransfected with KRC and various deletion constructs of the IL-2 promoter. In initial experiments, KRC transactivated a luciferase reporter driven by only 200 bp of the IL-2 proximal promoter. The most prominent regulatory sequences in this region are cis elements that bind members of the NFAT, NFκB, and AP-1 transcription factor families (Jain, J., C., *et al.* (1995) *Curr. Biol.* 7:333-342; Ullman, K.S., *et al.* (1993) *Genes & Development* 7:188-196; Rooney, J.W., *et al.* (1995) *Immunity* 2:473-483; Durand, D.B., *et al.* (1987) J. Exp. Med. 165:395-407), although the NFAT and NFκB cis elements have been shown to overlap. Therefore, whether KRC could transactivate a multimerized linked NFAT/AP-1 target site, or individual multimerized NFAT or AP-1 target sites was tested. KRC enhanced PMA/ionomycin-induced transactivation of a multimerized linked NFAT/AP-1 element and the isolated, multimerized AP-1 element but not the NFAT element (*Figure 19(C)*). In contrast to AP-1, the PMA/ionomycin induced activity of NFAT was not further increased by coexpression of KRC. KRC therefore acts at the transcriptional level to increase expression of IL-2 through an AP-1-site-dependent mechanism. Preliminary results show that KRC overexpression enhances, and KRC deficiency decreases, stimulation-induced upregulation of CD69 another AP-1 target gene in T cells (Castellanos, M.C., *et al.* (1997) *J. Immunol.* 159: 5463-5473).

EXAMPLE 20: KRC DOES NOT MODULATE MAPK ACTIVITY

In this example, the results demonstrate that KRC does not modulate MAPK activity. It was unlikely that KRC, a zing finger protein, transactivated the IL-2 promoter through direct binding to the AP-1 element, especially given the observation that KRC was able to enhance AP-1 activity only when Jurkat cells were simultaneously stimulated through the TCR pathway by PMA or antigen/APC. Indeed in EMSA assays using extracts prepared from unstimulated Jurkat cells overexpressing KRC, no binding to a radiolabeled AP-1 site oligonucleotide was detected. Thus, KRC and AP-1 do not bind the same site within the IL-2 promoter to synergistically increase promoter activity. Additionally, we observed that KRC does not increase AP-1 activity by increasing the expression of c-Jun/c-Fos mRNA

An alternative explanation was that KRC acts upstream to enhance posttranslational modifications of AP-1 that increase its activity. For example, N-terminal phosphorylation of c-Jun or C-terminal phosphorylation of c-Fos have been shown to enhance AP-1 activation downstream of the Ras pathway (Dumitru, C.D., *et al.* (2000) *Cell* 103:1071-1083; Binetruy, B., *et al.* (1991) *Nature* 351:122-127; Deng, T., and M. Karin (1994) *Nature* 371:171-175). Overexpression of a dominant negative Ras blocks TCR-induced AP-1 activity (Rayter, S.I., *et al.* (1992) *Embo J.* 11:4549-4556). More recently, it has been shown that mice deficient in PKC theta show defective TCR induced AP-1 activation, suggesting a role for this kinase in Ras/MAPK/AP-1 activation (Sun, Z., *et al.* (2000) *Nature* 404; Isakov, N., and A. Altman (2002) *Annu. Rev. Immunol.* 20:761-794). Both rottlerin, a PKC theta inhibitor, and overexpression of dominant negative Ras (RasN17) abolished the ability of KRC to enhance AP-1 transactivation following PMA/ionomycin stimulation (*Figure 20 (A)*). These data are consistent with the placement of KRC downstream of the Ras pathway or with a requirement for two distinct, but interconnected signals for IL-2 promoter transactivation. The latter explanation is more likely since KRC can increase AP-1 activation by Ras but cannot activate AP-1 on its own. Thus, KRC activation of AP-1 requires Ras, and KRC can substantially augment AP-1 activation by the Ras pathway.

KRC may enhance AP-1 function indirectly through the modulation of MAPK activity, kinases downstream of Ras that are known to potently stimulate AP-1 function (Binetruy, B., *et al.* (1991) *Nature* 351:122-127; Deng, T., and M. Karin (1994) *Nature* 371:171-175; Murphy, L., *et al.* (2002) *Nat. Cell Biol.* 4: 556-564). In T cells, stimulation via the TCR or with PMA/ionomycin induces the activation of three MAPKs: ERK, p38 and JNK. The activation of these MAPKS is required for AP-1 transcriptional activity. JNK, in particular, has been shown to increase AP-1 transcriptional activity by phosphorylating c-Jun (Arias, J., *et al.* (1994) *Nature* 370:226-229). In initial experiments it was determined that KRC overexpression did not alter levels of transcripts encoding a series of MAP3, MAP2 and MAP kinases as assessed by RNase protection assays (Pharmingen). To test whether KRC had any effect on MAPK activity, a sensitive assay, the PathDetect reporting system, was utilized to evaluate the effect of KRC on ERK-mediated ELK-1 transactivation and p38-mediated ATF2 transactivation. Jurkat cells were co-transfected with a pGAL4-UAS-LUC reporter and

expression plasmids encoding GAL4-Elk1 and GAL4-ATF2 fusion proteins, respectively. KRC was unable to modulate either MAPK or p38 activity in this assay (*Figure 20 (B)*). Co-expression of KRC with HA-ERK1, myc-ERK2, Flag-P38 and Flag-JNK2 was performed and the activity of each kinase was measured using an immunoprecipitation-kinase assay with specific substrates, GST-Elk1, GST-ATF2 and GST-Jun for each MAPK. KRC had no detectable effect on any of the MAPKs in this assay (results for JNK shown, *Figure 20 (C)*). Therefore, KRC does not increase AP-1 activity through increasing TCR mediated MAPK activity, although it was observed that KRC downregulates TRAF2-mediated JNK activation following TNFα stimulation in macrophage cell lines (Oukka, M., *et al.* (2002) *Mol. Cell* 9:121-131). Since PMA/ionomycin is a very poor inducer of JNK activation in T cells, the possibility that KRC might also downregulate JNK in T cells under different circumstances cannot be ruled out (*e.g.*, CD28 stimulation). However, the ability of KRC to *inhibit* low levels of JNK activity following prolonged CD3/CD28 stimulation of naïve Thp cells is unlikely to account for its ability to dramatically enhance AP-1 function and IL-2 production.

EXAMPLE 21: KRC PHYSICALLY INTERACTS WITH c-Jun AND ACTS AS A TRANSCRIPTIONAL COACTIVATOR

In this example, the results demonstrate that KRC physically interacts with c-Jun and acts as a transcriptional coactivator. It has been demonstrated that KRC interacts with the adapter protein TRAF2 to inhibit both NFκB and JNK/SAPK mediated responses including apoptosis and TNFα cytokine gene expression (Oukka, M., et al. 2002. *Mol. Cell* 9:121-131). To investigate whether KRC might therefore physically associate with c-Jun, expression vectors encoding c-Jun and a truncated myc-tagged version of KRC encoding amino acids 204 to 1055 (KRC tr), which includes the third zinc finger domain, one of the three acidic domains and the putative NLS sequence were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-myc antibody revealed that KRC physically associated with c-Jun (*Figure 21(A)*). Further, it demonstrated that the region of KRC shown to associate with TRAF2 (aa 204-1055) also interacted with c-Jun. Similar results were obtained in coimmunopreciptations of overexpressed full-length KRC with c-Jun, although the absolute amounts of c-Jun obtained were less, presumably because the full-length KRC

protein is poorly expressed due to its large size (*Figure 21 (B)*). Further mapping of c-Jun to delineate its interaction site with KRC revealed that KRC interacts with c-Jun amino acids 1-224 fused to the DNA binding domain of GAL4, which includes the transactivation domain Further, this association is direct and does not require posttranslational modifications as shown by the interaction of in vitro translated KRC and c-Jun proteins (*Figure 21 (B)*, right panel). Finally, it was important to demonstrate that this association occurred under physiologic conditions. Untransfected Jurkat or EL4 T cell lines were stimulated with PMA/ionomycin for 45 minutes, and AP-1 complexes were purified by immunoprecipitating c-Jun. *Figure 21 (C)* shows that endogenous KRC is readily detected in these complexes obtained from stimulated cells.

To further investigate the mechanism via which KRC serves as an AP-1 coactivator, AP-1 was activated by overexpressing c-Jun or c-Jun and c-Fos in 293T cells with an AP-1 luciferase reporter. In this system, overexpression of KRC enhances both c-Jun and c-Jun plus c-Fos AP-1 activity (approximately 5 fold, *Figure 21 (C)*). However, the presence of endogenous AP-1 proteins might complicate interpretation of these results. Therefore the Gal4 DNA binding domain was fused to the c-Jun or c-Fos transactivation domains and cotransfected these chimeric cDNAs with KRC and a Gal4 binding site-luciferase reporter construct into 293T cells. The chimeric GAL4-c-Jun, but not GAL4-c-Fos, protein potently transactivated the reporter construct in the presence of KRC demonstrating that KRC indeed acts as a transcriptional coactivator (*Figure 21(D)*). In sum then, KRC specifically associates with c-Jun under physiologic conditions and this association augments AP-1 transcriptional activity.

EXAMPLE 22: KRC PHYSICALLY ASSOCIATES WITH c-Jun BUT NOT c-Fos

In this example, the results demonstrate that KRC physically interacts with c-Jun but not c-Fos. Expression vectors encoding c-Jun, c-Fos and a truncated myc-tagged version of KRC encoding amino acids 204 to 1055 (KRC tr) which includes the third zinc finger domain, one of the three acidic domains and the putative NLS sequence were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-myc antibody revealed that KRC physically associated with the c-Jun/c-Fos AP-1 complex. Further, it demonstrated that

the region of KRC, aa 204-1055 shown to associate with TRAF2 also interacted with AP-1. KRC appeared to interact with both members of the AP-1 complex. However, 293T cells express endogenous c-Jun. To test definitively whether KRC interacted with both members of AP-1, in vitro translated c-Fos, c-Jun and KRCtr were coimmunoprecipitated using antibodies to c-Jun, c-Fos and KRC. In this assay KRCtr interacted with c-Jun but not c-Fos. Further, the interaction between KRCtr and c-Jun required only the c-Jun N-terminal portion AA 1-79, termed the delta domain. It was possible that posttranslational modification of c-Fos was required for its interaction with KRC. Alternatively, KRC might interact with c-Fos only when it was associated with c-Jun. Indeed, when c-Jun was present in the lysates, c-Fos coimmunoprecipitated with KRCtr. These experiments revealed that KRC physically associated with c-Jun, but not c-Fos, the high affinity association of c-Fos with endogenous c-Jun presumably leading to the coimmunoprecipitation of c-Fos with KRC observed above. Consistent with this result was the failure to detect association of KRC with c-Fos in a yeast two hybrid assay

EXAMPLE 23: KRC REGULATES THE STABILITY OF THE c-Jun/c-Fos AP-1 TRANSCRIPTION FACTOR THROUGH CONTROLLING ITS DEGRADATION

In this example, the results demonstrate that KRC regulates the stability of the c-Jun/c-Fos AP-1 transcription factor by controlling its degradation. The above experiments mapped the interaction site of KRC with c-Jun to aa 204-1055 of KRC. The interaction of full-length KRC with c-Jun was tested. However, attempts to demonstrate that full-length KRC interacted with AP-1 in overexpression experiments resulted in coimmunoprecipitation of very small amounts of c-Jun and no detectable c-Fos protein when compared to truncated KRC. These results raised the possibility that association of full-length KRC protein with AP-1 might lead to its degradation. Time course experiments were performed in which overexpressed sense KRC or an antisense KRC previously shown to block production of endogenous KRC protein were coimmunoprecipitated with overexpressed c-Jun and c-Fos. Overexpression of full-length KRC, in the presence of low dose cycloheximide to block endogeneous protein synthesis led to the rapid degradation of c-Jun (*Figure 22(B)*). Conversely,

overexpression of antisense KRC, by inhibiting the expression of endogenous KRC, decreased the rate of c-Jun degradation (*Figure 22(B)*). The same set of experiments were performed using c-Fos, a very short-lived cellular protein. As with c-Jun, the stability of the c-Fos protein in the presence of cycloheximide was compromised in the presence of KRC and dramatically stabilized in the presence of the KRC dominant negative expressing only the ZAS2 domain or in the presence of the antisense KRC (*Figure 22(A)*). Remarkably, degradation of c-Fos was almost completely abolished in the presence of antisense KRC, suggesting that KRC may be the major protein that controls c-Fos degradation in vivo. The ability of KRC to promote the degradation of other fos family members Fra1, Fra2 and Fos B was also tested (*Figure 23(D)*). Only c-Fos protein stability was deceased in the presence of KRC demonstrating the specificity of KRC for the c-Jun/c-Fos AP-1 pair. Viral Fos, an oncogene in acutely transforming retroviruses, contains a frameshift mutation that replaces the last 48 amino acids of c-Fos with an unrelated 49 amino acid-long C terminal tail that renders v-Fos a more stable protein compared to c-Fos. The increased stability accounts in part for the superior transformation ability of v-Fos. The protein stability of V-fos was not affected by altering levels of KRC by sense or antisense overexpression.

EXAMPLE 24: KRC REGULATES THE STABILITY OF THE c-Jun AND c-Fos BASED ON THEIR FUNCTION AS TRANSCRIPTIONAL ACTIVATORS

In this example, the results demonstrate that the effect of KRC in regulating the stability of c-Jun and c-Fos proteins is reflected in their ability to function as transcriptional activators. To examine the functional consequences of AP-1 degradation by KRC, cotransfection experiments in 293T cells with sense or antisense KRC together with a luciferase-tagged AP-1 reporter construct were performed. Overexpression of sense KRC resulted in decreased stimulation of AP-1 activity while conversely, expression of antisense or DN KRC led to an increase in AP-1 activity. To determine whether KRC alters both the level of activation per cell and the number of cells in which activation or repression occurs we used an AP-1 target site construct fused to GFP. Cotransfection of the AP-1-GFP construct together with KRC or antisense KRC into 293 cells revealed that KRC reduced both the number of cells in which GFP was expressed as well as the intensity of GFP expression per cell. Conversely, cotransfection

of antisense KRC increased AP-1 transactivation as evidenced by an increased number of GFP+ cells as well as an increase in the intensity of fluorescence per cell in. Thus, the effect of KRC in regulating the stability of the c-Jun and c-Fos proteins is reflected in their ability to function as transcriptional activators.

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EXAMPLE 25: KRC IS REQUIRED FOR UBIQUINATION OF BOTH c-Jun AND c-Fos

In this example, the results demonstrate that KRC is required for ubiquitination of both c-Jun and c-Fos. Much attention has recently been focused on the 10 role of covalent modification in controlling gene transcription in eukaryotes. Lysine modification by ubiquitination, sumoylation and acetylation of transcription factors contributes to their function in modulating gene expression. Previous studies have established that AP-1 proteins are rapidly degraded by the ubiquitin/ proteasome pathway. In this pathway, ubiquitin (UB) a 76 amino acid polypeptide is activated by the 15 formation of a thiol ester linkage by the ubiquitin activating enzyme (E1) and is then transferred to the active site cysteine of a ubiquitin carrier protein (E2). Formation of an isopeptide bond between the C terminus of UB and lysines on a substrate is catalyzed by a UB ligase (E3), which binds the substrate and catalyzes the transfer of the UB from a specific E2 to the substrate. The formation of a chain of UB molecules on the substrate 20 then targets it for degradation by the 26 S proteasome. It has been shown that KRC interacts with AP-1 to regulate its degradation raising the possibility that KRC might be the elusive AP-1 E3 UB ligase responsible for its ubiquitination in vivo.

EXAMPLE 26: KRC KNOCKOUT B CELLS HAVE IMPAIRED IgA 25 PRODUCTION AND TGFβ-DEPENDENT GLα TRANSCRIPTION

Homozygous mutant KRC KO mice have normal lymphocyte development as determined by FACS analysis of primary and secondary lymphoid organs. Despite normal B cell development, analysis of serum immunoglobulins (Igs) in non-immunized KRC KO mice revealed a selective reduction of circulating IgA (Figure 30 23A). The decrease in serum IgA correlated with observations *in vitro* that purified splenic CD 19+ B cells from KRC KO mice, activated under conditions that promote

IgA class switching, secreted significantly lower levels of IgA than WT B cells (Figure 23B).

To determine if KRC regulates IgA production at the level of transcription, Iga germline transcripts (GLα) in activated WT and KRC KO B cells were analyzed. Consistent with the decreased IgA secretion observed, activated KRC KO B cells had a marked reduction in levels of GLα transcripts when compared to WT B cells (Figure 23C). It has previously been reported that TGFβ signaling in B cells has a central role in regulating GLα transcription through SMAD3 and Runx3 mediated processes (Zhang, Y., and Derynck, R. 2000. J Biol Chem 275: 16979-16985; Shi, M. J., and Stavnezer, J. 1998. J Immunol 161: 6751-6760.).

Given the findings that KRC can interact with SMAD3, it was determined whether KRC could augment the transcriptional activity of SMAD3 and/or Runx3 in driving the expression of GLα. A luciferase reporter plasmid driven by the mouse GLα promoter (-179/+46) was cotransfected into the Ml2 B-cell line along with KRC, Runx3 and/or SMAD3 expression constructs. Cotransfection of KRC enhanced the ability of SMAD3 and Runx3 to drive the expression of the reporter plasmid (Figure 23D). M12 cells express endogenous SMAD3 and therefore it is not clear if the effects of KRC on Runx3 may be independent of SMAD3. Therefore KRC regulates IgA class switching as well as other B cell effector functions by acting downstream of the TGFβ receptor in these cells.

Signaling by Decapentaplegic (Dpp), a member of the TGFß superfamily of signaling molecules similar to vertebrate BMP2 and BMP4, has been implicated in many developmental processes in Drosophila melanogaster. Notably, Dpp acts as a long-range morphogen during imaginal disc growth and patterning. Genetic approaches led to the identification of a number of gene products that constitute the core signaling pathway. Decapentaplegic (Dpp) signaling leads to association of Medea (Med) with Mothers against *dpp* (Mad) Mammalian homologues of the Drosophila Med and Mad proteins are the SMADs. Once Dpp associates with Med and Mad, it then translocates to the nucleus where it interacts with Schnurri. In addition to Schnurri, Dpp signaling and Brinker (Brk), to prime cells for Dpp responsiveness.

It has been demonstrated that Schnurri is required for Dpp-mediated gene repression. It was therefore determined whether KRC could interact with the mammalian homologue of Mad, SMAD3. KRC physically interacts with two R-SMADs, SMAD3 and to a lesser extent with SMAD2 but does not interact with the Co-SMAD, SMAD4 (Figure 23E). This is consistent with what has been observed in Drosophila, where Shn interacts with Mad but not Med. In addition, it was found that KRC enhances the transcriptional ability of SMAD3 to drive expression of a luciferase reporter construct containing a basic SMAD-binding element (Figure 23F).

EXAMPLE 27: KRC AUGMENTS Th2 CYTOKINE PRODUCTION AND INTERACTS WITH GATA3

Composite AP-1/NFAT sites are found in the proximal promoter regions of many cytokine genes such as TNFα, GM-CSF, IL-2, IL-3, IL-4, and IL-5 (Rao, A. 1994. Immunology Today J_5: 274-281; Rooney, J. et al. 1995. Immunity 2: 473-483.). Given that KRC is an inducible AP-1 coactivator for the IL-2 gene, it was determined whether KRC could regulate other AP-1 -dependent genes in T cells. A systematic analysis of cytokine production by primary lymph node (LN) and splenic CD4+ T cells stimulated by plate-bound anti-CD3/CD28 in the absence of polarizing cytokines (unskewed conditions) from KRC WT and KO mice was performed. As was previously published, KRC KO CD4+ cells showed a striking defect in IL-2 production at early time points (up to 36 hours) (Oukka, M., et al. 2004. J Exp Med 199: 15-24.). Additionally, KRC KO T cells displayed reduced proliferation at early time points compared to WT cells, as measured by ^{3}H incorporation. This proliferation defect was completely rescued by the provision of exogenous hIL-2, indicating that it was due completely to reduced IL-2 production. Moreover, analysis of IL-2 production by real-time PCR and ELISA at later time points of primary stimulation showed that KRC KO T cells produced levels of IL-2 equivalent to WT cells at all time points during primary anti-CD3/CD28 stimulation beyond 36 hours showing redundancy by other Schnurri family members. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 72 hours in the presence of 200 U/ml human IL-2. Supematants were analyzed for IFNy, IL-4, and IL-5 levels by ELISA. For subsequent experiments described below, exogenous hIL-2

was added to all cultures to account for any early differences between WT and KRC KO CD4+ T cells. Analysis of Th effector cytokine production revealed dramatic differences between KRC WT and KO cells following 72 hours of primary unskewed stimulation. As shown in Figures 24A and 24B, while KRC WT and KO cells secreted similar levels of the Thl effector cytokine IFNγ, production of Th2 effector cytokines IL-4 and IL-5 was drastically reduced in KRC KO cells despite normal proliferation, indicating that the defective Th2 cytokine production was not due to decreased cell division.

To investigate the consequences of decreased IL-4 and IL-5 in these unskewed primary stimulations, LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 72 hours in the presence of 200 U/ml human IL-2 (unskewed). Cells were expanded for an additional 4 days in the presence of hIL-2 and restimulated with plate-bound anti-CD3. As expected, production of all Th2 effector cytokines was dramatically reduced in secondary stimulations of KRC KO cells, as shown in Figure 24C. However, when cells were initially stimulated under Th2-polarizing cytokines (IL-4 plus neutralizing antibodies to IFNγ), production of Th2 effector cytokines by KRC KO cells was identical to WT cells (Figure 24D). LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 ug/ml) for 72 hours in the presence of hlL-2, IL-4 and neutralizing antibodies to IFNγ (Th2-skewed). Cells were expanded for 3 days in hlL-2, and restimulated for 18 hours with plate-bound anti-CD3 (2 μg/ml). Supematants were analyzed for IL-4, IL-5, IL-6, IL-10, and IL-13 levels by ELISA. These results indicated that KRC KO cells were not defective *per se* in producing Th2 cytokines; rather, KRC was required for the establishment of the Th2 effector cell under unskewed primary stimulation conditions. Given that KRC mRNA is rapidly induced in Thp cells following TCR/CD28 ligation (Oukka, M., et al. 2004. J Exp Med 199: 15-24) and that KRC mRNA levels fall 2-3 days following primary T cell activation, it shows that KRC induction plays a role in reinforcing the activity of factors required for Th2 cell generation. Moreover, since KRC KO cells produce perfectly normal levels of IL-2 at later time points, and KRC KO Th2 effector cells secrete normal levels of all AP-1-dependent Th2 cytokines, these results strongly suggested that KRC's role in Th2 cell generation was independent from its ability to function as an AP-1 coactivator.

To further analyze the defect in Th2 cell generation in the absence of KRC, RNA and cDNA were prepared from WT and KRC KO CD4+ T cells at 0, 12, 24, and 48 hours following anti-CD3/CD28 stimulation in unskewed conditions. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for the indicated times in the presence of 200 U/ml human IL-2. RNA and cDNA were made and analyzed for the presence of IL-4 and GATA3 mRNA relative to fi-actin using real-time PCR. Levels of IL-4 and GATA3 transcripts were analyzed by real time PCR. As shown in Figure 24E, although initial induction of IL-4 mRNA was comparable between WT and KO cells, KRC KO cells were unable to fully upregulate IL-4 following 48 hours of CD3/CD28 stimulation. Strikingly, this defect in production of high levels of IL-4 mRNA was accompanied by nearly absent upregulation of the Th2-specific transcription factor GATA3 at these time points (Figure 24F).

Since the defect in GATA3 upregulation preceded the defect in IL-4 upregulation, the primary lesion in Th2 cell generation in the absence of KRC was in the early induction of GAT A3. Therefore, WT and KRC KO cells were transduced with control GFP and bicistronic GFP-GATA3 retroviruses 24 hours following primary TCR/CD28 stimulation in unskewed conditions. Cells were then expanded in hIL-2, restimulated with PMA/ionomycin, and assayed for secondary Th2 cytokine production by intracellular cytokine staining. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 24 hours in the presence of hIL-2. Cells were then infected with retroviruses expressing either GFP or GFP-GATA3. Cells were expanded in hIL-2 for 3 days, and subsequently restimulated with PMA/ionomycin for 6 hours. Intracellular cytokine staining to analyze IL-4 production in GATA3-negative and GATA3-positive cells was performed. As expected, GFP-negative and control GFP-expressing KRC KO cells showed reduction in intensity of IL-4 and absolute cell number of IL-4 producers (Figure 24G). Additionally, although levels of GFP were identical between WT and KO RV-GATA3 cultures, KRC KO RV-GATA3 -expressing cells failed to express levels of IL-4 comparable to WT RV-GATA3-expressing cells. These results indicated that KRC lay both upstream and downstream of GATA3 in its ability to regulate the generation of IL-4-producing Th2 cells.

In addition to its ability to directly transactivate the IL-5 and IL-13 genes and to induce chromatin remodeling of the entire Th2 cytokine locus, another well-documented property of GATA3, like many 'master regulator' transcription factors, is its ability to auto-activate itself (Ouyang, W., et al. 2000. Immunity 12: 27-37). Since both GATA3 induction and GATA3 activity were reduced in KRC KO cells, KRC plays a role in directly regulating the function of GATA3, in its ability to auto-activate itself and/or in its ability to drive activation of the Th2 cytokine locus. Since KRC can interact with SMAD3 (Figure 23) and SMAD3 can bind and potentiate GATA3-driven transcription (Blokzijl, A., et al. 2002. Curr Biol 12: 35-45), KRC could regulate GATA3 activity by binding GATA3 itself. 293T cells were transfected with KRC with or without FLAG-GATA3. 48 hours later, cells were lysed and FLAG-tagged proteins were immunoprecipitated overnight with anti-FLAG beads. Immunoprecipitates were washed, resolved by SDS-PAGE, and KRC was detected by immunoblotting. As shown in Figure 24H, when overexpressed in 293T cells, FLAG-GATA3 specifically precipitated overexpressed KRC. To evaluate the function of this physical interaction, the ability of overexpressed KRC to regulate GATA3-driven transcription from an IL-5-luciferase construct (Miaw, S. C, et al. 2000. Immunity 12: 323-333) was tested in EL4 cells. As shown in Figure 24I, while KRC had no effect on IL-5-driven transcription in the absence of co-expressed GATA3, the combination of KRC and GATA3 led to dramatic enhancement of GATA3 transcriptional activity, consistent with the previously-described role for KRC as a transcriptional coactivator. Note that neither Shn-1 nor Shn2 could augment GATA3-dependent IL-5 promoter activation. Finally, to determine whether KRC could potentiate GATA3-driven auto-activation of the GATA3 gene itself, the ability of KRC to potentiate GATA3's ability to drive activation of different segments of the GATA3 genomic locus fused to luciferase was tested (Hwang, E. S., et al. 2002. J Immunol 169: 248-253). Much like its ability to potentiate GATA3's activity on the IL-5 promoter, KRC also strongly enhanced the ability of GATA3 to drive expression from a previously described intronic enhancer between exons 1 and 2 of the GATA3 locus (Figure 24J). EL4 cells were electroporated with 1 μg IL-5-luciferase reporter (G) or 1 ug of GATA3-luciferase reporters (H) with the indicated combinations of GATA3 (4 μg) and Shns 1,2 and 3 (20 μg). 18 hours later, cells were stimulated with PMA/ionomycin for 6 hours and luciferase activity was determined.

EXAMPLE 28: KRC DEGRADES ITS PARTNERS

In the course of mapping the interaction site of KRC with c-Jun, it ws observed that coimmunoprecipitation of full-length KRC with c-Jun in overexpression experiments resulted in very small amounts of c-Jun and no detectable c-Fos protein when compared to truncated KRC. These results raised the possibility that association of full-length KRC protein with its partners might lead to their degradation. As shown in Figure 25, experiments in which KRC was coexpressed with c-Jun (upper left), c-fos (upper right), SMAD3, Runx2, GATA3 and TRAF2 (bottom) were performed. 293T cells were transiently transfected with c-Jun (upper left), c-Fos (upper right), or FLAG-tagged Smad3, Runx2, Gata3, and Traf2 (bottom) with or without KRC. 48 hours later, cells were treated with 10 ug/ml cycloheximide for 15 minutes. Whole cell lysates were prepared and 30 ug protein/sample was resolved by SDS-PAGE followed by immunoblotting for c-Jun, c-Fos, or FLAG. Blots were stripped and reprobed with anti-Hsp90 antibody as a loading control. Overexpression of full-length KRC in the presence of low dose cycloheximide to block endogeneous protein synthesis led to the rapid degradation of all of these proteins. However, KRC augments cJun, SMAD3 and GATA3-dependent gene activation despite its ability to degrade these transcription factors. Ubiquitination of transcription factors leads to their degradation but also can increase their potency in transactivation simultaneously with their degradation (Molinari, E., et al. 1999 Embo J 18:6439-6447; Salghetti, S. E., et al. 2001. Science 293: 1651-1653; von der Lehr, et al. 2003. Mol Cell H: 1189-1200; Grossman, S. R., et al. 2003. Science 300: 342-344; Greer, S. F., et al. 2003. Nat Immunol 4: 1074-1082.). Further, inclusion of the proteasome inhibitor MG-132 prevented the degradation of Fos by KRC (upper right). Therefore, KRC might be an E3 ubiquitin ligase.

EXAMPLE 29: KRC UBIQUITINATES ITS PARTNERS, TRAF2 AND Runx2

It is known that KRC physically associates with the above transcription factors, and that this association results in the degradation of these proteins. One major pathway for protein degradation is the ubiquitin/protesasome complex. In preliminary ubiquitination assays, increased ubiquitination of two KRC partners, TRAF2 and Runx2, was detected, demonstrating that KRC functions as a component of an E3 ligase (Figure

26). These experiments were performed by transiently transfecting 293T cells with FLAG-tagged Runx2 or Traf2 with or without KRC. 48 hours later, cells were treated with 10 ug/ml cycloheximide for 15 minutes. Whole cell lysates were prepared and 30 ug protein/sample was resolved by SDS-PAGE followed by immunoblotting for FLAG. Blots were stripped and reprobed with anti-Hsp90 antibody as a loading control. 293T cells were transiently transfected with FLAG-tagged Runx2 or Traf2 with the indicated combinations of His-Ubiquitin and KRC. 48 hours later, cells were treated with the proteasome inhibitor MG132 (10 uM) for 2 hours. Cells were lysed in 6M guanidium-Hcl, and His-ubiquitin-conjugated proteins were precipitated with Ni-NTA agarose. Precipitates were washed and resolved by SDS-PAGE followed by immunoblotting anti-FLAG to detect poly-ubiquitinated Runx2 or Traf2 species.

The functional outcome of TRAF2 and Runx2 degradation is straightforward since KRC actually represses TRAF2 and Runx 2 driven responses in vitro (Oukka, M., Kim, et al. Mol Cell 9: 121-131).

EXAMPLE 30: Shn2 and KRC HAVE OVERLAPPING BUT UNIQUE FUNCTIONS

Mice that lack Shn2 have severely impaired positive selection of CD4+ and CD8+ cells, and peripheral CD4 T cells had impaired production of IL-2 (Takagi, T., et al. 2001. Nat Immunol 2: 1048-1053). The mechanism by which Shn2 acts to control these functions has not been established. In order to determine how Shn2 controls these T cell functions, Jurkat cells were electroporated with 2 ug 2xAP-1-luciferase reporter along with 20 ug vector, Shn2, or KRC DNA. Eighteen hours later, cells were stimulated with PMA/ionomycin for 6 hours and luciferase activity was determined. Figure 27 shows that like KRC, Shn2 associates with AP-1 to transactivate an AP-1 reporter. However, Shn2 does not coactivate SMAD3 or GATA3's (Figure 24G) ability to transactivate the GLα or IL-5 genes, respectively, in the absence or presence of TGFβ.

EXAMPLE 31: PHENOTYPIC ANALYSIS OF KRC KNOCKOUT ANIMALS

The most pronounced immune system abnormalities of these mice are evidence of impaired TGFβR signaling in B cells and impaired early development of the T helper 2 (Th2) lineage from its progenitor (Thp), i.e. KRC KO Th cells have impaired

production of Th2, but not Th1 cytokines. Analysis of serum Igs in KRC KO mice as well as *in vitro* secretion of Igs by KRC KO B cells has also revealed a role for KRC in the regulation of IgA production.

The generation and regulation of effector B cell functions involve a complex temporal network of cytokines, signaling proteins, and transcription factors. Dysregulation of any one component may compromise the B cell's ability to mediate its effector functions and contribute to a failure of the host immune system to effectively respond to foreign pathogens. As described above, deletion of KRC results in impaired IgA secretion and transcription of the GLα gene in vivo.

TGFβ has been demonstrated to influence various aspects of normal B cell biology and is important in regulating humoral immune responses. In normal cells, TGFβ signaling is initiated when this molecule binds to and induces a heterodimeric cell-surface complex consisting of type I (TbRI) and type II (TbRII) serine/threonine kinase receptors. This heterodimeric receptor then propagates the signal through phosphorylation of downstream target SMAD proteins. There are three functional classes of SMAD protein, receptor-regulated SMADs (R-SMADs), Co-mediator SMADs (Co-SMADs) and inhibitory SMADs (I-SMADs). Following phosphorylation by the heterodimeric receptor complex, the R-SMADs complex with the Co-SMAD and translocate to the nucleus, where in conjunction with other nuclear proteins, they regulate the transcription of target genes (Derynck, R., et al. (1998) Cell 95: 737-740).

Mice with a B-cell specific inactivation of TβRII have increased B-cell responsiveness, enhanced antibody production and a selective defect in the production of antigen-specific IgA (Cazac, B. B., and Roes, J. (2000) Immunity 13: 443-451). Further analysis of TGFβ signaling in B cells has demonstrated that this cytokine can modulate the expression of approximately 100 different genes in B cells (Roes, J., et al. (2003) Proc Natl Acad Sci U S A 100: 7241-7246). TGFβ can elicit different cellular responses in B cells through its ability to positively and negatively regulate gene transcription. Both activation and repression of gene expression by TGFβ utilize the same set of ubiquitous SMAD proteins. However, specific cofactors that bind to SMADs are believed to dictate whether a gene is upregulated or downregulated in response to TGFβ (Shi, Y., and Massague, J. (2003) Cell 113: 685-700). A similar transcriptional mechanism may account for the variable effects of TGFβ on B-cell effector function. Identification

of the different cofactors expressed in B cells will be critical to fully understand how TGFβ regulates B cell function.

Disruption of those molecular pathways that regulate B cell function may also contribute to the development of B-cell leukemia and lymphomas. Most lymphoid neoplasms have chromosomal translocations or mutations that allow them to bypass the normal cellular checkpoints that control their propagation. During normal physiological processes, TGFβ serves as a potent negative regulator of cell growth and differentiation, thus serving as a key tumor suppressor. Several hematopoietic neoplasms, including B cell chronic lymphocytic leukemia (B-CLL), have genetic alterations that impair TGFβ signaling in these cells and render them nonresponsive to the growth-inhibiting effects of TGFβ (Schiemann, W. P., et al. (2004) Cancer Detect Prev 28: 57-64).

No role for the mammalian Shn genes in TGFβ signaling has yet to be identified although the three known vertebrate Shn orthologs have been postulated to be downstream of the bone morphogenetic protein-transforming growth factor-beta-activin signaling pathways (Rusten, T. E., et al. (2002) Development 129: 3575-3584). Given the well-defined role of Drosophila Shn in regulating Dpp, it was determined whether KRC is a component of the TGFβ signaling pathway. Indeed, it has been demonstrated that KRC physically interacts with two R-SMADs, SMAD3 and to a lesser extent with SMAD2, but does not interact with the Co-SMAD, SMAD4. This is consistent with what has been observed in Drosophila, where Shn interacts with Mad but not Med. In addition, KRC enhances the transcriptional ability of SMAD3 to drive expression of a luciferase reporter construct containing a basic SMAD-binding element.

KRC is not downstream of TGFβR in T cells but that it is downstream of the TGFβR in osteoblasts as well as in B cells. A profound abnormality in development of the skeletal system is present in KRC KO mice. These mice exhibit an osteosclerotic phenotype that is characterized by increases in trabecular bone mass, bone mineral density and bone formation consistent with impaired signaling through the TGFβ receptor. While SMAD3 and the transcription factor Runx3 interact to activate transcription of the GLα gene, SMAD3 and another Runx family member, Runx2 act to repress transcription of the osteocalcin gene. KRC interacts with all three transcription factors. However, while KRC is a coactivator of GLα promoter activity, it is a

corepressor of the osteocalcin gene. Hence, in its absence, GLα transcription is diminished in B cells but osteocalcin gene transcription is augmented in osteoblasts.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.